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Ribonucleic Acid Metabolism in the Liver after Administration of Individual Amino Acids

By H. N. MUNRO and D. MUKERJI
Department of Biochemistry, The University, Glasgow

(*Received* 9 *December* 1957)

The effects of protein deficiency on the metabolism of ribonucleic acid in the liver have already been described (Munro, Naismith & Wikramanayake, 1953; Clark, Naismith & Munro, 1957). The amount of ribonucleic acid in the liver falls rapidly during the first day of protein depletion and then becomes adjusted at a lower level. During the initial phase of reduction, uptake of ^{32}P and of [^{14}C]glycine by ribonucleic acid is greatly diminished. This is probably due to breakdown products of ribonucleic acid causing dilution of labelling among the precursors used for its synthesis. Feeding of protein prevents this dilution effect, as is evidenced by the rapid restoration of labelling of ribonucleic acid. This suggests that the breakdown is partly or wholly suspended while dietary amino acids are being absorbed from the gut. These findings led us to conclude that the stability of liver ribonucleic acid is determined by the supply of amino acids for synthesis of protein. Consequently, it was of interest to know whether the metabolism of liver ribonucleic acid is affected by feeding with a protein deficient in one or more essential amino acids. This question has now been examined by using zein as the test protein. Contrary to expectation, zein was found to be almost as effective as casein in obliterating evidence of breakdown of ribonucleic acid. Extension of these studies to mixtures of amino acids confirmed that changes in metabolism of ribonucleic acid are not dependent on all essential amino acids being fed simultaneously. Individual amino acids were then administered singly, and it was found that glycine, methionine and leucine were each capable of producing a considerable effect on the metabolism of liver ribonucleic acid. The high leucine content of zein is sufficient to account for a large part of its action on the metabolism of ribonucleic acid.

EXPERIMENTAL METHODS

Animals and diets. Male albino rats weighing about 180–200 g. were housed in separate cages under thermostatic conditions. They were fed on a synthetic diet (Munro & Naismith, 1953) in two portions: the morning meal provided vitamins, minerals, 4·3 g. of carbohydrate and 0·2 g. of fat, and the evening meal contained 1·3 g. of carbohydrate, 0·5 g. of fat and all the dietary protein (2·2 g. of casein). The rats quickly learned to consume these meals promptly. After about 3 or 4 days on the diet the test protein or amino acid was substituted for the casein of the evening meal; control animals received only the carbohydrate and fat of this meal. The few animals which did not eat the final meal promptly were rejected. The remainder received 40 μc of ^{32}P by intramuscular injection 1 hr. later. They were killed the next day, 18 hr. after injection. Thus the period of labelling coincided with absorption of the final test meal.

Removal and analysis of liver. The rats were killed by exsanguination. After perfusion with 0·9% NaCl, the liver was excised and analysed for ribonucleic acid phosphorus (RNA P) and in some cases protein N by procedures described previously (Clark *et al.* 1957). These constituents are expressed as mg./100 g. body wt., in order to indicate the total amount in the liver corrected for small variations in the initial size of the animals used.

The specific activities of the inorganic phosphate of the liver and of the individual ribonucleotides of ribonucleic acid (RNA) were measured by the procedures used earlier (Clark *et al.* 1957). In the tables, the specific activity of RNA P is given as a percentage of the specific activity of the inorganic phosphate fraction of the same liver (relative specific activity). In order to estimate the total incorporation into RNA P per liver, the total relative activity (Campbell, Olley & Blewett, 1949) was computed. This was obtained by multiplying the total amount of RNA P in the liver (mg./100 g. body wt.) by the percentage figure obtained for relative specific activity.

Test protein and amino acids. Zein, the only nutritionally incomplete protein studied, was obtained commercially and gave a negative glyoxylic acid test. The casein used was unextracted grade (Glaxo Laboratories Ltd., Greenford, Middlesex).

The amino acid mixtures used in the experiments reported in Table 2 were compounded in the proportions found to be optimum for rat growth by Rose, Oesterling & Womack (1948). Two mixtures were given, one consisting of all the essential amino acids except tryptophan, and the second containing only non-essential amino acids. Rats fed on the former mixture received 100 mg. of L-arginine hydrochloride, 40 mg. of L-cystine, 190 mg. of L-histidine hydrochloride, 240 mg. of DL-isoleucine, 240 mg. of L-leucine, 300 mg. of L-lysine hydrochloride, 160 mg. of L-methionine, 240 mg. of L-phenylalanine, 140 mg. of L-threonine, 120 mg. of L-tyrosine and 200 mg. of L-valine per rat. The latter mixture provided 40 mg. of L-alanine, 800 mg. of L-aspartic acid, 1200 mg. of L-glutamic acid, 400 mg. of glycine, 40 mg. of L-proline and 400 mg. of DL-serine per rat. $NaHCO_3$ was added where necessary to neutralize acidic amino acids.

The significance of the high leucine content of zein was investigated by feeding with a mixture of amino acids in the proportions found in that protein (Block & Bolling, 1951). Each rat received 216 mg. of L-alanine, 34 mg. of L-arginine hydrochloride, 105 mg. of L-aspartic acid, 19 mg. of L-cystine, 501 mg. of L-glutamic acid, 32 mg. of L-histidine hydrochloride, 138 mg. of L-isoleucine, 439 mg. of L-leucine, 44 mg. of L-methionine, 121 mg. of L-phenylalanine, 196 mg. of L-proline, 57 mg. of L-threonine, 2 mg. of L-tryptophan, 98 mg. of L-tyrosine, 146 mg. of DL-serine and 57 mg. of L-valine. A control series of rats received an additional 298 mg. of L-alanine in place of the 439 mg. of leucine.

Individual amino acids were usually given in 1 g. quantities, because this was generally the maximum amount which most animals would voluntarily consume in the evening meal. All were commercial preparations, the glycine (British Drug Houses Ltd.) being of AnalaR quality. The L-leucine (British Drug Houses Ltd.) was stated to contain traces of L-isoleucine; estimation of its sulphur content (Young, Edson & McCarter, 1949) showed that it could not have been significantly contaminated by methionine. The action of L-leucine on metabolism of RNA is unlikely to be due to contaminants, since synthetic DL-leucine has a similar effect (Table 4).

Dose–response curves with individual amino acids. For three amino acids, the response of metabolism of RNA to different doses was investigated on rats of closely similar weight. With glycine, the mean body weight at different dose levels varied from 202 to 209 g., with methionine from 202 to 210 g., and with leucine from 175 to 188 g. The doses administered were 0·05, 0·1, 0·2, 0·4, 0·6 and 1·0 g. of glycine/rat, 0·05, 0·1, 0·2, 0·4 and 0·7 g. of methionine/rat and 0·1, 0·3, 0·5 and 1·0 g. of leucine/rat.

RESULTS

In the first 24 hr. after protein has been withdrawn from the diet of the rat, there is a sharp decrease in the RNA content of the liver. Previous experiments (Munro *et al.* 1953; Clark *et al.* 1957) have shown that incorporation of ^{32}P and of [^{14}C]glycine into RNA is much reduced during this period; these changes were rapidly reversed by feeding with casein just before injection of the isotopes. In the present series of experiments, a nutritionally incomplete protein has been used for feeding under the same circumstances, to see whether deficiency of essential amino acids might lead to a different response in metabolism of RNA from that evoked by casein. Table 1 shows that, as observed in the earlier studies, inclusion of casein in the meal fed just before injection of isotope produced a large increase in uptake of ^{32}P by RNA (relative specific activity and total relative activity). However, the feeding with zein had an effect which was statistically indistinguishable from that of casein and, moreover, the addition of the missing amino acids tryptophan and lysine to the zein did not alter the magnitude of the response.

In view of this finding with zein, mixtures of amino acids were tested. Table 2 shows that a mixture made up of all the essential amino acids except tryptophan increased total relative activity by 47 %, and a mixture of non-essential amino acids raised it by 136 % above the level obtained by feeding with the protein-free meal alone. The latter figure exceeds the effect of 2·2 g. of casein on uptake of ^{32}P by RNA. When the individual non-essential amino acids were given singly in 1 g. doses (Table 3), glycine was the only component of the mixture which caused measurable changes in metabolism of RNA. In a further experiment (not reported here) it was found that the uptake of ^{32}P was unaffected by aspartic acid and glutamic acid even in doses of 2·2 g./rat. A dose–response curve was constructed for glycine over the range 0·05–1·0 g./rat (Fig. 1). The metabolism of RNA was not significantly affected until 0·4 g. of glycine had been added to the protein-free meal; this distinctive pattern appeared in each repetition of the experiment and indicates a threshold dose for stimulation by glycine.

The essential amino acids (including cystine and tyrosine) were then given singly in 1 g. doses

Table 1. *Liver ribonucleic acid metabolism after feeding with casein or zein*

Animals received a final meal of carbohydrate and fat to which was added the substance tested; 40 μc of ^{32}P was injected 1 hr. after the final meal and the animals were killed 18 hr. later. The mean results from four experiments are shown. All additions to the diet significantly increased total relative activity ($P < 0{\cdot}01$), but the increment after feeding with casein was not significantly greater than that after zein fed alone or supplemented with tryptophan and lysine. Total relative activity is explained in Experimental Methods.

		Uptake of ^{32}P by RNA P		
Addition to final meal	RNA P/100 g. body wt. (mg.)	Relative specific activity (%)	Total relative activity	Change in total relative activity (%)
None	3·13	17·4	0·54	—
Casein (2·2 g.)	3·12	38·1	1·18	+119
Zein (2·2 g.)	3·35	29·9	1·00	+85
Zein (2·2 g.) + 0·05 g. of tryptophan + 0·1 g. of lysine	2·94	33·3	1·01	+87

Table 2. *Liver ribonucleic acid metabolism after administration of essential and non-essential amino acid mixtures*

Animals received a final meal of carbohydrate and fat to which was added the substance tested; 40 μc of ^{32}P was injected 1 hr. after the final meal and the animals were killed 18 hr. later. The figures are the means of two experiments. The feeding with casein and with the non-essential amino acid mixture raised total relative activity significantly ($P<0.05$).

		Uptake of ^{32}P by RNA P		
Addition to final meal	RNA P/100 g. body wt. (mg.)	Relative specific activity (%)	Total relative activity	Change in total relative activity (%)
None	2·98	19·4	0·58	—
Casein (2·2 g.)	3·73	34·1	1·27	+119
Essential amino acid mixture (2·0 g.)	3·69	23·3	0·85	+47
Non-essential amino acid mixture (2·9 g.)	4·09	33·5	1·37	+136

Table 3. *Liver ribonucleic acid metabolism after administration of individual non-essential amino acids*

Animals received a final meal of carbohydrate and fat to which was added the amino acid. The amino acids were used in 1 g. quantities. 40 μc of ^{32}P was injected 1 hr. after the final meal and the animals were killed 18 hr. later. The figures are the means of two experiments. There are no statistically significant differences in total relative activity except with glycine ($P<0.01$).

		Uptake of ^{32}P by RNA P		
Addition to final meal	RNA P/100 g. body wt. (mg.)	Relative specific activity (%)	Total relative activity	Change in total relative activity (%)
None	3·40	22·2	0·73	—
L-Glutamic acid	3·16	24·3	0·77	+6
L-Aspartic acid	3·26	25·6	0·84	+16
DL-Serine	3·39	24·6	0·84	+16
DL-Alanine	3·56	22·3	0·79	+9
L-Proline	2·78	24·2	0·68	−6
Glycine	3·87	34·2	1·30	+80

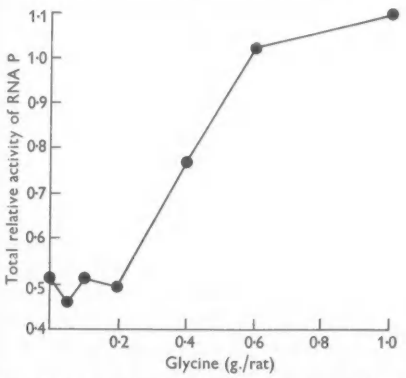

Fig. 1. Uptake of ^{32}P by liver ribonucleic acid (total relative activity) after administration of different doses of glycine to rats of uniform size. The rats received a meal of carbohydrate and fat to which the glycine was added. 40 μc of ^{32}P was injected 1 hr. after the meal and the animals were killed 18 hr. later. Each point is the mean from two experiments. The action of glycine on total relative activity is highly significant ($P<0.01$).

(Table 4). In this instance, there was a less clear-cut distinction between amino acids affecting RNA metabolism and those not affecting it. Statistically significant differences between treatments which would correspond to 1 and 5% probability levels were established by analysis of variance of the results as a whole. After two amino acids, L-methionine and DL-leucine, total relative activity was greater than that of the control group by a difference which exceeded the 1% probability level. Feeding with L-cystine and with L-tryptophan caused increments reaching the 5% probability level, but with cystine this effect was due to one exceptionally large figure, and with tryptophan the response just attained the 5% probability level. It was considered that only with methionine and leucine were the effects on RNA metabolism sufficient to justify further exploration. Dose–response curves for L-methionine and L-leucine were therefore constructed (Figs. 2, 3). In contrast with glycine (Fig. 1), both amino acids produced a response in metabolism of RNA which was approximately linear over the dose range studied. All rats receiving more than 0·2 g. of methionine

Table 4. *Liver ribonucleic acid metabolism after administration of individual essential amino acids*

Animals received a final meal of carbohydrate and fat to which was added the amino acid. The amino acids were used in 1 g. quantities. 40 μc of ^{32}P was injected 1 hr. after the final meal and the animals were killed 18 hr. later. For convenience of handling, the amino acids were divided into two arbitrary groups. Figures are the means of three experiments. Statistical analysis shows a highly significant difference between animals fed with methionine or leucine and the control group ($P<0.01$). With cystine and tryptophan, the increment is just significant ($P<0.05$).

Addition to final meal	RNA P/100 g. body wt. (mg.)	Uptake of ^{32}P by RNA P: Relative specific activity (%)	Uptake of ^{32}P by RNA P: Total relative activity	Uptake of ^{32}P by RNA P: Change in total relative activity (%)
None	3·47	21·2	0·75	—
DL-Threonine	3·02	18·9	0·58	−23
L-Lysine	3·29	24·5	0·80	+7
L-Histidine	3·38	26·8	0·92	+23
L-Phenylalanine	3·43	28·4	0·97	+30
L-Valine	4·04	25·6	1·02	+36
None	3·24	19·4	0·62	—
DL-Isoleucine	2·94	18·6	0·56	−10
L-Arginine	3·11	24·0	0·74	+19
L-Tyrosine	3·51	22·3	0·79	+27
L-Tryptophan	3·43	26·6	0·91	+47
L-Cystine	3·87	25·8	0·97	+56
DL-Leucine	4·11	25·4	1·04	+68
L-Methionine	3·63	33·3	1·21	+95

in the final meal showed considerable gastric retention when killed 18 hr. later.

From these experiments it is apparent that glycine, methionine and leucine each have a considerable effect on uptake of ^{32}P by liver RNA. Tables 3 and 4 show that, associated with this action, there is a small increment in the amount of RNA in the liver. To evaluate this change statistically, all available experiments involving these three amino acids have been collected into Table 5. In each instance, the animals receiving the amino acid have more RNA/liver than similar control animals.

The final experiment deals with the application of these findings to the action of zein on metabolism of RNA. According to published analyses (Block & Bolling, 1951) zein contains 23·7 % of leucine, 2·3 % of methionine and no glycine. In order to determine whether the high leucine content of zein might account for its stimulant action on metabolism of RNA, rats were fed with a mixture of amino acids in the same proportions as they occur in zein. A control group received the same mixture with all the leucine replaced by an equimolar quantity of alanine. Table 6 shows that the mixture containing leucine increased uptake of ^{32}P by 77 %, which is comparable with the action of zein (Table 1). However, the corresponding mixture without leucine produced a 36 % increment above the basal level, which indicates that the other amino acids made some contribution to the response. This is not likely to be due to the small amount of methionine in the mixture, since reference to the dose–response curve for methionine

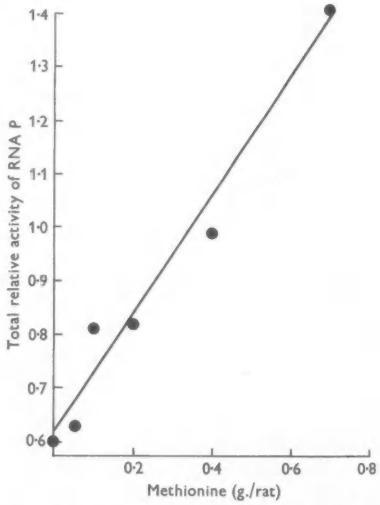

Fig. 2. Uptake of ^{32}P by liver ribonucleic acid (total relative activity) after administration of different doses of L-methionine to rats of uniform size. The rats received a meal of carbohydrate and fat to which the methionine was added. 40 μc of ^{32}P was injected 1 hr. after the meal and the animals were killed 18 hr. later. Each point is the mean from two experiments. The data are described by the equation $Y=0.62+1.09X$, where X is the dose of methionine, in g./rat, and Y represents total relative activity. The regression coefficient is highly significant ($P<0.01$).

(Fig. 2) shows that a dose of 44 mg./rat would not produce a measurable increase in total relative activity.

In the same experiment, the protein content of the liver was also examined (Table 6). The stimulus to metabolism of RNA is accompanied by a greater amount of protein in the liver, but the effect fails to attain statistical significance. However, significant changes were observed in the experiment in which the response of metabolism of RNA to increasing doses of L-leucine was explored (Fig. 3). At dose levels of 0, 0·1, 0·3, 0·5 and 1·0 g. of leucine, the average protein content of the livers was 64, 65, 68, 70 and 72 mg. of N/100 g. of body wt. respectively. The correlation coefficient for these data is +0·60, which is significant ($P = 0·05$–$0·02$).

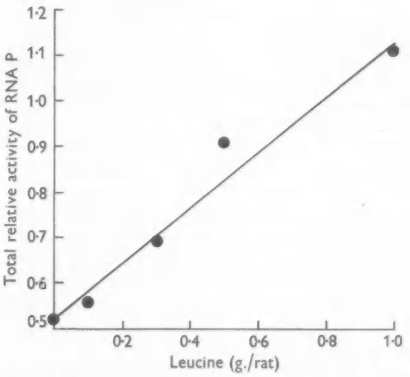

Fig. 3. Uptake of ^{32}P by liver ribonucleic acid (total relative activity) after administration of different doses of L-leucine to rats of uniform size. The rats received a meal of carbohydrate and fat to which the leucine was added. 40 μc of ^{32}P was injected 1 hr. after the meal and the animals were killed 18 hr. later. Each point is the mean from three experiments. The data are described by the equation $Y = 0·52 + 0·62X$, where X is the dose of leucine, in g./rat, and Y represents total relative activity. The regression coefficient is highly significant ($P < 0·01$).

DISCUSSION

In an earlier study (Clark *et al.* 1957), it was concluded that the metabolism of ribonucleic acid in the liver is influenced by the dietary supply of amino acids available for synthesis of protein. The present investigation was begun in order to test the response of the metabolism to the feeding of proteins deficient in one or more essential amino

Table 5. *Changes in the amount of liver ribonucleic acid after administration of individual amino acids*

Animals received a final meal of carbohydrate and fat to which 1 g. of the amino acid was added. Control rats were given the protein-free meal alone. All rats were killed 19 hr. later. Statistical analysis shows that each amino acid increased the amount of RNA/liver significantly ($P < 0·01$ for glycine, $P < 0·05$ for methionine and for the combined results for leucine).

		RNA P/100 g. body wt.		
Substance administered	No. of comparisons	Control series (mg.)	Animal fed with amino acid (mg.)	Change (%)
Glycine	5	3·34	3·84	+15
L-Methionine	6	3·13	3·56	+14
L-Leucine	8	2·79	2·98	+9
DL-Leucine	7	3·11	3·49	

Table 6. *Liver ribonucleic acid metabolism after administration of an amino acid mixture resembling zein in composition*

Animals received a final meal of carbohydrate and fat to which was added the amino acid mixture. A control group received the mixture with all the leucine replaced by alanine. 40 μc of ^{32}P was injected 1 hr. after the final meal and the animals were killed 18 hr. later. The figures are the means of five experiments. Both amino acid mixtures increased total relative activity significantly ($P < 0·01$ for the mixture containing leucine; $P = 0·02$–$0·01$ for the mixture without leucine).

		Uptake of ^{32}P by RNA P			
Addition to final meal	RNA P/100 g. body wt. (mg.)	Relative specific activity (%)	Total relative activity	Change in total relative activity (%)	Liver protein N/100 g. body wt. (mg.)
None	2·86	15·3	0·42	—	69
Amino acid mixture (2·2 g.)	3·23	23·8	0·74	+77	78
Amino acid mixture less leucine (2·0 g.)	2·91	21·0	0·57	+36	74

acids. It is apparent from our findings with zein that factors other than the biological value of a protein can determine its effect on metabolism of ribonucleic acid in the liver. Certain individual amino acids, notably glycine, methionine and leucine, stimulate uptake of ^{32}P considerably, and the action of zein is largely attributable to its high leucine content (Table 6).

The significance of this action of individual amino acids on metabolism of ribonucleic acid has still to be explored. We can exclude the possibility that the observed changes are artifacts of isotope incorporation arising from distortion of the product–precursor relationship; after feeding with glycine, methionine or leucine there was a significant increase in the total amount of ribonucleic acid in the liver (Table 5). The changes in metabolism of ribonucleic acid which follow feeding with these three amino acids are probably secondary to alterations in the rate of liver-protein synthesis. In leucine-fed rats there is an accumulation of protein in the liver in parallel with the changes in uptake of ^{32}P (Fig. 3), and on the basis of our earlier investigations (Clark *et al.* 1957) we can interpret this to mean that leucine produces an increase in the rate of protein synthesis leading to greater incorporation of isotope into ribonucleic acid. Although the liver protein of glycine-fed and methionine-fed rats was not measured, it is probable that the action of these two amino acids on metabolism of ribonucleic acid can be similarly explained. Even if we accept this explanation, the mechanism underlying the increase in the protein content of the liver remains obscure. It may depend on an increased secretion of hormones from the adrenal cortex. There is evidence that perfusion of the eviscerated dog with solutions of certain amino acids leads to an increase in the secretory activity of the cortex (Vogt, 1951), and it has been conclusively established that one action of cortisone is to cause protein to accumulate in the liver (see review by Trémolières, Derache & Lowy, 1955).

The nutritional significance of our findings has still to be evaluated. The addition of excessive amounts of individual amino acids to diets gives rise to amino acid imbalances and toxicities (see review by Harper, 1956), and our observations are presumably related to such effects. When zein is the sole protein of the diet, the action of leucine does not result in any permanent rise in the amount of protein in the liver. Campbell & Kosterlitz (1948) studied the non-glycogen non-lipid solids of liver (essentially a measure of liver-protein content) during the first 3 days of feeding with a diet containing, as protein source, zein supplemented with lysine. On the first day the level of liver solids was much higher than on the first day of a protein-free diet, but by the third day the group fed on zein had lost this advantage. This suggests that the action of zein on the protein content of the liver may occur only before protein depletion has set in.

SUMMARY

1. The uptake of ^{32}P by liver ribonucleic acid has been studied after administration of certain proteins, amino acid mixtures and individual amino acids.

2. Feeding with the nutritionally incomplete protein zein resulted in the same increase in ^{32}P uptake as did the feeding with casein. Addition of tryptophan and lysine to zein did not alter the magnitude of the response.

3. Eighteen amino acids were used for feeding singly in large doses and it was established that glycine, methionine and leucine each caused a significant increment in uptake of ^{32}P. The responses after the feeding of methionine and of leucine were proportional to the dose administered, whereas the response to glycine occurred only after a threshold dose had been reached.

4. Experiments with a mixture of amino acids in the proportions found in zein confirmed that its high leucine content can account for a large part of its action on metabolism of ribonucleic acid.

5. The protein content of the liver is increased by administration of leucine, and it is concluded that the changes in metabolism of ribonucleic acid after the feeding of glycine, methionine and leucine are secondary to an increased rate of synthesis of liver protein.

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The Enzymic Degradation of Starch by Holotrich Protozoa from Sheep Rumen

By D. L. MOULD and GWEN J. THOMAS*
Rowett Research Institute, Bucksburn, Aberdeenshire

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It has been shown that the holotrich ciliates found in the rumen of sheep fed on a starch-free diet can store large amounts of iodophilic granules (Masson & Oxford, 1951). This material was identified as starch rather than glycogen since it gave an insoluble iodine complex under the conditions described by Pucher, Leavenworth & Vickery (1948). Further evidence was given by Forsyth & Hirst (1953) who proved by methylation studies that the storage material was an amylopectin having an average chain length of 25 glucose units. Some experiments with β-amylase and with the debranching enzyme R-enzyme, to be described below, also point to the similarity between this storage material and potato amylopectin.

During the course of experiments designed to distinguish between several mechanisms which might be involved in the synthesis of amylopectin by the protozoan organisms, it became apparent that the search for the synthesizing enzyme was being complicated by the presence of a highly active amylase in the cell extract. The purification of this amylase, and its separation from the other carbohydrases found in the Protozoa, are described in this paper. During fractionation by salt-precipitation methods or by the use of specific absorbents a certain amount of activity was lost (cf. Bernfeld, 1955). Since differences between the isoelectric points of amylases and invertases exist (Dieu, 1947; Bernfeld, Duckert & Fischer, 1950; Fischer, Kohtès & Fellig, 1951; Fischer & de Montmollin, 1951*a*) it was thought that fractionation of the mixture of amylase, maltase, β-glucosidase, invertase and the starch-synthesizing enzyme present in the cell contents of the Protozoa might be achieved by use of the methods of preparative zone electrophoresis (Tiselius & Flodin, 1953). The method has been partially successful, with the result that a reversible phosphorolytic enzyme system similar to that existing in the higher plants and animals has been found to operate in the protozoan cell. Also of interest is the separation of three specific amylases present in aqueous extracts of holotrich Protozoa. The amylolytic action of two of the amylases has been determined, and an approximate estimate of their electrophoretic mobility and isoelectric points has been made.

* Present address: Lister Institute for Preventive Medicine, London.

MATERIALS AND METHODS

Preparation of holotrich material

Phosphate–acetate buffer. The composition of this was (%, w/v): NaCl 0·5, sodium acetate 0·13, KH_2PO_4 0·03, K_2HPO_4 0·10, $MgSO_4,7H_2O$ 0·01. The solution was adjusted to pH 7·2 with dilute NaOH.

Citrate buffer. Sodium citrate (0·2 M) was adjusted to pH 5·5 with 5 N-NaOH.

Separation of holotrich ciliates from rumen fluid. The method was essentially that of Heald & Oxford (1953). A sample of rumen contents (usually 1 l.) was taken with tube and suction bulb 2 hr. after the sheep had been fed. The sample was strained through six layers of surgical gauze, and allowed to stand at 39° for 1 hr. Half the supernatant was sucked off, and the bottom layer, which contained Protozoa and debris, was put into a separating funnel. Glucose dissolved in phosphate–acetate buffer was added to give a final concentration of 0·75%, and the funnel was placed in the incubator. The white bottom layer, settling during fermentation of glucose, was withdrawn at intervals into boiling tubes filled with the buffer at pH 7·2. Several washings by decantation were required to remove the scum and debris which rose to the surface with the gas produced by the holotrichs. In those cases where it was intended to test the Protozoa for phosphorylase activity the final washings were carried out with citrate buffer. The Protozoa were then transferred into screw-capped bottles and stored at −20° until required.

Separation of Isotricha *and* Dasytricha. Microscopic observation of the ciliates collected as above showed that the rumen holotrichs *Dasytricha rumiantium*, *Isotricha prostoma* and *I. intestinalis* were invariably present, although the proportion of *Isotricha* and *Dasytricha* varied to a marked extent. *Isotricha* and *Dasytricha* were separated by a modification of the technique of Gutierrez (1955). The Protozoa which had been drawn off the base of the separating funnel were divided into six portions, each of which was poured on top of separate liquid columns containing clarified rumen liquor (38 ml.), phosphate–acetate buffer (15 ml.) and glycerol (3 ml.). Protozoa which settled during the first 10 min. were the heavier *Isotricha*, and these were drawn off and washed with buffer in the usual manner. *Dasytricha*, which remained suspended in the column, were deposited during centrifuging and, after several washings with buffer, were stored at −20°. Overloading of the columns led to poor separation of the Protozoa since some *Dasytricha* were brought down with the *Isotricha*. Portions

of each fraction drawn off the columns were examined under the microscope, and those tubes containing only *Isotricha* were kept, and their contents pooled. Gutierrez's method of isolating the *Dasytricha* which remained as streaks on the separating funnel after the rumen contents had been drained out was not adopted as the yield obtained in this way was too low.

Counting the Protozoa. Counts of the Protozoa suspended in buffer–glycerol (1:1) were made in a cell described by Boyne, Eadie & Raitt (1957).

Preparation of cell-free filtrates. Protozoa which had been stored at −20° were quickly brought to room temperature by immersing the bottles in water at 25°. This caused the Protozoa to burst, thus liberating the starch granules. The cell debris and starch were removed by centrifuging, and the supernatant was dialysed in cellophan for 48 hr. at 2° with stirring against distilled water. In this way the enzymes were obtained free from starch dextrins and various sugars. The extract was freeze-dried, and could be stored for 6 months without loss in activity of the amylases.

Preparation of substrates and enzymes

Amylose. This was prepared from potato starch by the $Al(OH)_3$–thymol procedure of Hobson, Pirt, Whelan & Peat (1951).

Amylopectin. This was prepared by fractionation of thymol–amylopectin with the methanol procedure of Hobson *et al.* (1951).

Holotrich starch. The starch granules were liberated from the ciliates as described above. Much of the cell debris was removed by light centrifuging, which left the starch in suspension. The starch was further purified by the technique of Sevag (1934). A solution of the starch was shaken with $CHCl_3$ and amyl alcohol for 24 hr. in a centrifuge tube. The mixture was centrifuged for 30 min. and the top layer containing the starch was removed. The starch was precipitated with 3 vol. of ethanol. The purity as determined by acid hydrolysis and estimation of reducing sugar was 97%.

Grass fructan. Italian ryegrass was cut before the flowering stage and air-dried at 100°. The milled grass was extracted in a Soxhlet thimble with 85% (v/v) ethanol for 8 hr. This treatment removed all pigments, hexoses, sucrose, raffinose and some short-chain polyfructosans. The grass residue was shaken with water overnight, filtered on a Büchner funnel, and the filtrate treated with 4 vol. of ethanol. The precipitated fructan was dissolved in water and desalted by passage through ion-exchange columns packed with Amberlite IR-120 (H) and -4B (OH). The deionized fructan was again precipitated with ethanol, and dried over P_2O_5 *in vacuo* at room temperature. The product had $[\alpha]_D$ −42°. (R_F=0 in propanol–ethyl acetate–water solvent mixture, 6:1:3, by vol.)

Salivary α-amylase. The method of preparation of Meyer, Fischer, Staub & Bernfeld (1948) was followed to the stage before crystallization.

R-enzyme. This was prepared from broad beans as described by Hobson, Whelan & Peat (1951).

β-Amylase. Pure β-amylase was prepared from soya beans by the method of Peat, Pirt & Whelan (1952).

Measurement of enzyme activity

Amylase. A solution of potato amylose (0·2%) was prepared by dispersing amylose wetted with ethanol in 0·1 N-NaOH and heating at 100° for 5 min. After it had been cooled and neutralized with 0·1 N-H_2SO_4, the solution was appropriately diluted. The volume added to the digest mixture was arranged to bring the final concentration of amylose to 1 mg./ml. In most cases a 6 ml. digest was prepared, containing 1 ml. of 0·2 M-citrate buffer (pH 5·5). The amount of enzyme added was such that the fall in light absorption of the polysaccharide–iodine solution after 15 min. at 35° was not more than 7 scale units on the EEL colorimeter (Evans Electroselenium Ltd.) with 1·3 cm. diam. cells and Ilford filter no. 608 (max. transmission 680 mμ) when a sample (1 ml.) of the digest was withdrawn and made up to 100 ml. with I_2 (2 mg./100 ml.) and KI (20 mg./100 ml.) for measurement of EV (Hobson & Macpherson, 1952); EV is the light-absorption measured on the EEL colorimeter. One activity unit was defined as the amount of amylase which produced a fall in EV of one colorimeter scale unit under the above conditions.

β-Glucosidase, invertase and maltase. Digests of total volume 6 ml. contained 1 ml. of citrate buffer (pH 5·5) and the concentration of substrate was usually 0·3% for maltose and cellobiose, 2% for sucrose and 1·5% for fructan. Portions (1 ml.) were withdrawn at intervals for the determination of reducing power with the Somogyi (1945) reagent. The increase in reducing power during the incubation was a measure of the activity of the enzyme.

Phosphorylase. The activity was determined by measuring the amount of glucose 1-phosphate formed in a digest containing amylose and phosphate. Amylose (140 mg.) was dissolved by heating in 0·1 N-NaOH and brought to neutrality with N-H_2SO_4. Ammonium molybdate (8·3%, w/v; 8 ml.) and 0·002 M-$HgCl_2$ (0·6 ml.) were added and the solution was made up to 50 ml. with phosphate buffer (30 ml.). The phosphate buffer contained KH_2PO_4 (13·617 g.) and Na_2HPO_4 (35·995 g.) in 1 l., giving a concentration of 6·5 mg. of P/ml.

Digests containing amylose solution (3 ml.) and phosphate buffer (2 ml.) were prepared and brought to 35°. Enzyme samples (2 ml.) were then added and the digest was incubated at 35° for 20 hr. Excess of inorganic phosphate was removed by precipitation with magnesia reagent and centrifuging. The magnesia reagent contained $MgCl_2$ (14 g.), NH_4Cl (17 g.) and strong aq. NH_3 soln. (sp. gr. 0·880; 30 ml.) in 250 ml. of solution. After centrifuging, determinations of free inorganic phosphate and of 7 min.-hydrolysable phosphate were made by the method of Allen (1940). The difference between these represented the amount of glucose 1-phosphate present, and this was related to the activity of the enzyme.

Zone electrophoresis

Zone electrophoresis was carried out in starch columns according to the method of Flodin & Porath (1954) (see also Flodin & Kupke, 1956). The holotrich amylases were strongly adsorbed to cellulose and cellulose derivatives, as was discovered during preliminary studies of the mobility of the amylases on filter-paper strips or cellulose columns. No tailing of the enzyme preparations was, however, observed in starch columns and there was full recovery of enzyme activity in the eluate. Crystalline salivary α-amylase was strongly adsorbed to the starch columns. This may possibly be due to an insolubility effect and surface denaturation of the pure preparation. A preparation of *Streptococcus bovis* amylase appeared to be unaffected by elution through a packed-starch column, but the activity of

the preparation was too low for a satisfactory investigation of the trailing edge of the eluted zone.

Apparatus. Commercial potato starch was sieved and the fraction passing through a 300-mesh sieve was used as the packing material for the column. It was washed and treated with buffer as described by Flodin & Porath (1954). The column consisted of a glass tube 55 cm. long × 3 cm. diam. with an outer cooling jacket along its whole length, through which water from a thermostatic bath (10–25°) was circulated. The starch packing was supported above the glass filter at the base of the column by a close-packed glass-wool plug. The total length of the packed column was 48·5 cm., the effective cross-sectional area 2·4 cm.^{2} and the ratio starch vol./mobile liquid vol. was 2·0. The hold-up volume was 116 ml. The general construction of the apparatus was, with a few modifications, in principle the same as that described by Flodin & Porath (1954). Large silver–silver chloride electrodes were used sufficient for a total passage of 8000 c. A p.d. of 300 v was applied from a stabilized high-tension supply, and the duration of electrophoresis was 18–20 hr.

An appreciable electro-endosmotic flow is developed through a starch medium in the presence of phosphate buffers (Kunkel & Slater, 1952). For reproducible electrophoretic movement of zones in the column it was found necessary to leave the electrode vessels open and allow a head of buffer solution to be formed in the cathode vessel which could then act as a hydrostatic counterbalance for the electro-osmotic flow. The requisite difference in levels between the two electrode vessels at the commencement of electrophoresis for any set of experimental conditions could quickly be established by experience, e.g. a difference of 12·1 cm. was required with phosphate buffer, pH 7·0, I 0·05, electrophoresis current 13·7 mA and column temp. 20°. Volume changes due to electrode reactions occur slowly throughout the duration of electrophoresis but the auto-regulated head is able to adjust itself to these changes and remains constant, although the level in both electrode chambers may fall. Since the electrode vessels themselves were of large volume it was also advantageous to keep the temperature of the surroundings reasonably constant. All electrophoresis was carried out at an ambient temperature of 16–20°. The current remained constant to within 1 % in 24 hr. under these conditions. The anode was always connected to the bottom of the packed column. At the end of the electrophoresis the starch column was eluted with buffer at a flow rate of 15 ml./hr. and 4 ml. fractions were collected.

Buffer solutions. Sodium phosphate buffer, pH 7·0, I 0·05, was used throughout. Stock solutions of 0·2 M-Na_2HPO_4 (135 ml.) and 0·2 M-NaH_2PO_4 (95 ml.) were diluted to 2 l.

Preparation of enzyme solutions. Samples (100 mg.) of freeze-dried dialysed aqueous extracts of holotrich organisms were dissolved in phosphate buffer (2 ml.) and centrifuged at 14 000 g for 15 min. The clear supernatant solution, usually brown or straw-coloured, was applied to the column to form the initial zone (1·5–1·8 ml.). Samples of the supernatant solution were also taken for determination of enzyme activity.

Determination of enzyme activity. Amylase activity was determined by a similar method to that described above. The distribution of enzyme activity in the eluate fractions after electrophoresis was tested by taking samples (0·2–0·5 ml.) from each fraction according to the total activity applied to the column. In order to keep the sample volumes small and thereby conserve the amylase fractions the digests were incubated for 1 hr. at 35°. The digests consisted of 2 ml. of amylose solution (2 mg./ml.), 1·8 ml. of acetic acid–sodium acetate buffer, pH 4·8, and 0·2 ml. of enzyme solution. Measurement of EV was by the usual procedure (Hobson & Macpherson, 1952) and expressed in the scale units of an EEL colorimeter. Approximate estimates of total activities of the enzyme solutions were made by summation over the elution curves, assuming a linear relation in EV with enzyme concentration and one activity unit being defined as the amount of amylase which produced an EV of one colorimeter scale unit under these digest conditions.

Invertase, phosphorylase and other enzyme activities were determined as described above.

Dry-weight determinations. In certain fractionations, the distribution of total dry weight in the eluate from the starch columns after electrophoresis was measured. Eluate fractions (2 ml.) of known weight were collected in small weighing bottles and freeze-dried. Drying was completed to constant weight *in vacuo* over conc. H_2SO_4.

Ovalbumin preparation. A preparation of ovalbumin was used as a comparison material for estimating the electrophoretic mobility of the enzymes in the column. It was prepared from egg white by half-saturated $(NH_4)_2SO_4$ precipitation (Warner, 1954), and was recrystallized twice, dialysed and freeze-dried. The preparation contained both ovalbumin and conalbumin and separated into two zones of quite distinct mobility during zone electrophoresis in phosphate buffer on the starch column. The distribution of the protein in the eluate was observed by measuring the light-absorption of the fractions at 280 mμ in a 1 cm. cuvette on a Beckman DU quartz spectrometer.

Boundary electrophoresis. Analyses of the ovalbumin–conalbumin mixture and a holotrich enzyme preparation were made in phosphate buffer, pH 7·0, I 0·1, under standard procedure in a Hilger boundary electrophoresis apparatus by Dr E. I. McDougall.

RESULTS

Action of holotrich extract on amylose

When the cell-free extract was allowed to act on potato amylose the digest rapidly became achroic. The iodine stain of portions of the digest was tested at intervals, and the colour changes from blue through purple and red to orange were typical of those which occur during α-amylolysis. The products of the reaction, identified on a paper chromatogram, were glucose, maltose and maltotriose. This distinguished the enzyme from β-amylase, which would have produced maltose only. The relationship between absorption value and percentage apparent conversion into maltose was also typical of an α-amylase, the conversion at 10 % of the original absorption value being 18 %, and 30 % at the achroic point.

The optimum pH and temperature of action of the enzyme were determined, and found to be pH 5·5 and 50° respectively. The digests prepared

for the experiments were similar to the standard digests described above but sodium acetate–veronal buffer was used for the optimum pH experiment.

Effect of chloride and calcium ions on the amylase activity

Digests were set up containing $CaCl_2$ and NaCl to give a concentration of 0·0167 M. The enzyme was not activated by either salt, nor by 0·1 M-$MgSO_4$. Thus the amylase may be contrasted with salivary α-amylase, which is activated by 0·001 M-NaCl, and with malt α-amylase, which requires Ca^{2+} ions (Schwimmer & Balls, 1949; see Bernfeld, 1951).

Amylolytic degradation of holotrich starch

The reaction between holotrich extract and potato starch continued until the starch was completely converted into glucose. Holotrich starch was also completely degraded. This points to the presence in the extract of an amylo-1:6-glucosidase, and also a maltase since amylases have no action on maltose. Attempts were made to separate maltase from amylase by fractionation with ethanol, and with ammonium sulphate, but no substantial increase in the ratio amylase:maltase was obtained. There was, however, a large variation in the maltase activity of holotrich extracts made from rumen samples taken at different times.

When potato amylopectin and holotrich starch were incubated with salivary α-amylase, the reaction was arrested at 68 % conversion into maltose (Table 1). This limit was raised to 100 % if the amylase was allowed to act together with the debranching enzyme R-enzyme. The results in Table 1 show that holotrich starch and potato amylopectin are attacked in exactly the same way by R-enzyme and α-amylase. The simultaneous action of the two enzymes proceeds to a further extent than does the successive action, showing that R-enzyme cannot fully debranch amylopectin unless some of the α-1:4-glucosidic linkages between the branch linkages are hydrolysed by α-amylase. The characteristic increase in the iodine stain of amylopectin during incubation with R-enzyme was also observed when R-enzyme acted on holotrich starch.

Pure β-amylase prepared from soya beans hydrolysed holotrich starch to the extent of 51·5 % as maltose. The same result was obtained with starch prepared from *Isotricha*, and with starch from *Dasytricha*. Starch was also extracted from holotrich Protozoa which had been allowed to store polysaccharide at a lower temperature (25°) than usual, thus producing an abnormal appearance described by Eadie & Oxford (1955) in which the starch granules clump together in the middle of the cell. This starch was converted into maltose to the extent of 57 %. This 'abnormal' starch was similar to normal holotrich starch in other tests. Both starches exhibited behaviour similar to amylopectin during ultrafiltration in collodion membranes (Mould & Synge, 1954). After chromatography in formamide (Nussenbaum, 1951) both holotrich starches stained red with iodine and remained at the origin with no streaking. Amylose and soluble starch were used as controls. The wavelength of maximum absorption of the iodine–holotrich polysaccharide complex was also the same (540 mμ).

The simultaneous action of R-enzyme and β-amylase on holotrich starch gave a conversion into maltose of 100 %. Thus R-enzyme can hydrolyse all the branch linkages of holotrich starch after they have been exposed by β-amylase action. In this respect the starch differs from potato amylopectin, which has never been completely hydrolysed by R-enzyme plus β-amylase action, owing, it is thought, to the presence of phosphate groups which hinder the access of R-enzyme to some of the branch linkages (Peat, Whelan & Thomas, 1956). Waxy-maize starch, which has a negligible phosphorus content, is completely converted into maltose by the simultaneous action of R-enzyme and β-amylase (Thomas, 1952).

Electrophoretic separation of holotrich amylases

In preliminary experiments for the determination of optimum conditions for fractionation of the enzymes, zone electrophoresis was carried out in veronal buffer, pH 8·6 (I 0·1 or 0·05), and at

Table 1. *Enzymic degradation of amylopectin*

For conditions see text. The following abbreviations are used: α, salivary α-amylase; β, soya-bean β-amylase; R, R-enzyme; R, α, successive action of R and α; R + α, simultaneous action of R and α; R + β, simultaneous action of R and β.

	Apparent conversion into maltose (%)						
	Holotrich starch				Potato amylopectin		
Time (hr.)	α	R, α	R + α	R + β	α	R, α	R + α
2	32·8	65·8	67·7	63·2	29·4	71·9	73·9
8	42·2	73·8	81·9	81·9	51·2	75·9	81·3
22	60·6	78·0	86·1	88·4	61·9	82·5	85·3
70	68·3	86·5	98·1	101·5	64·5	87·8	101·2
99	66·4	86·8	99·4	—	68·1	—	101·8

temperatures 20–26° or 12–14°. Two well-defined zones of amylase activity were observed with an overlap of an invertase zone with the faster-moving zone. Recovery of amylase activity from the column was, however, only 50–70%, presumably due to the alkaline conditions. With phosphate buffer, pH 7·0 (I 0·05), and a column temperature 20°, total recovery of enzyme activity was achieved with good separation of the zones and these conditions were used throughout. With an applied p.d. of 300 v the current through the column was 13–14 mA and the time of electrophoresis 20 hr.

The distribution of amylase and invertase activity in the eluate fractions is shown in Fig. 1*a*. There are two well-separated peaks of amylase activity distinguished subsequently as A_D and A_I for the fast- and slow-moving components respectively. The invertase peak always overlapped the leading edge of A_D and has never been completely resolved. The proportion of total activity between the two amylase peaks varied considerably for different enzyme-extract preparations. The ratio A_D/A_I varied from 1·1 to 2·5 over eight separate preparations and in two extreme cases was 6·1 and 0·6. The actual height of the peaks depends on the sample volume and the original loading of the column.

The eluate fractions containing maximum amylase activity were pooled, freeze-dried and stored at 2°. The dried material was redissolved in a small volume of water and, after dialysis against phosphate buffer, reapplied to the column and electrophoresis was repeated under identical conditions. The enzyme activity was very much reduced after this procedure but it could be shown (Fig. 1*b*, *c*) that the enzyme fractions retained their characteristic mobility although there was extensive tailing of the slow-moving peak A_I.

A detailed examination of the total loss of activity of the fractionated enzymes during freeze-drying and dialysis was made. There was a 20% loss in invertase activity on freeze-drying in concentrated phosphate and a further loss amounting to 25% on prolonged dialysis against distilled water. The loss in activity on freeze-drying the amylase fractions could be as high as 40%, but they are fairly stable to extended dialysis against phosphate buffer (pH 7·0, I 0·02). Dialysis of the amylase solutions in the presence of 0·01 M-$CaCl_2$ gave similar results. Amylase A_D would appear to be more stable than A_I, which is in agreement with the general observed behaviour of fractionated and stored material. The enzyme stability is likely to be affected by partial purification and the adsorption tailing observed in the repeat electrophoresis of amylase A_I might be explained as arising from the deposition of insoluble enzyme on the surface of the supporting media. The unfractionated enzyme preparation from holotrich Protozoa was very stable towards dialysis and freeze-drying.

The amylolytic behaviour of the enzymes was investigated with the various peak eluate fractions from the column in the presence of phosphate buffer immediately after electrophoresis.

In order to assay further the degree of purification of the enzymes the distribution of total dry matter in the eluate fractions after electrophoresis was examined (Fig. 2). The dry solid content of the buffer in the column was 3·5 mg./ml. The two peaks of amylase activity lie on either side of quite a sharply defined peak of maximum dry solids. Amylase A_D is associated with the peak more than amylase A_I, which is apparently enriched. This is in agreement with the observed higher stability of the former enzyme during freeze-drying, storage

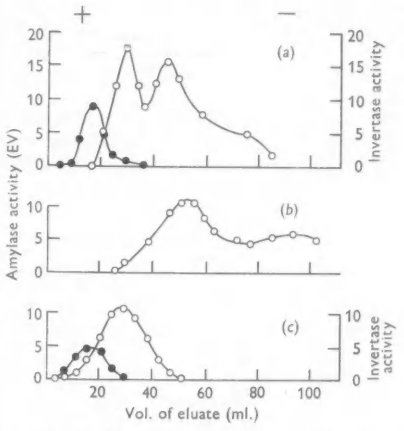

Fig. 1. Distribution of amylase and invertase activity in eluate from column after zone electrophoresis. Phosphate buffer, pH 7·0, I 0·05. (*a*) Mixed holotrich extract. (*b*) Repeat electrophoresis of material in slow-moving peak. (*c*) Repeat electrophoresis of material in fast-moving peak. ○, Amylase activity; ●, invertase activity.

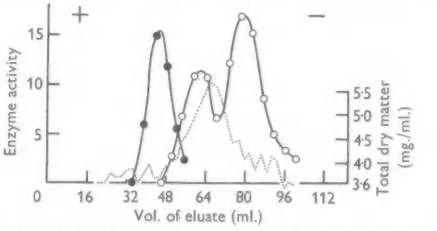

Fig. 2. Distribution of total dry matter and enzyme activity in eluate from column after zone electrophoresis of mixed holotrich extract. ○, Amylase activity; ●, invertase activity.

and dialysis. The dry solid content across the invertase peak is very low and the faster-moving portion of this peak must contain very enriched enzyme.

A rough estimation of the purification factor can be made from the data displayed in Fig. 2. The original activity of the invertase/mg., expressed in arbitrary units (see Methods), was 22. Assuming collection and pooling of the invertase fractions uncontaminated with amylase and no loss on freeze-drying and dialysis, the activity/mg. of the preparation would have been 656. The activity of the amylase (both types)/mg. in the original extract is 32. If fractions covering the whole A_I peak were collected and pooled, again assuming no loss of activity, the activity/mg. of the A_I preparation would have been 83.

Electrophoretic behaviour of amylases from Dasytricha *and* Isotricha

On the hypothesis that the enzymes A_I and A_D might be specific to either of the two types of Protozoa present, or that the ratio A_D/A_I might be specific to a particular organism, aqueous enzyme extracts from single organism preparations were subjected to zone electrophoresis.

The electrophoretic distribution of amylase activity due to *Isotricha* (Fig. 3) shows a small A_D component only, compared with A_I. During the sedimentation of the Protozoa from a suspension of filtered rumen contents after the addition of glucose, the larger *Isotricha* are deposited first, so that in normal preparations of mixed Protozoa it is highly probable that there will be a preponderance of *Isotricha* in the mixture. Owing also to the preferential sedimentation of *Isotricha*, there are difficulties in estimating visually the true distribution of organisms in the preparation (Boyne *et al.* 1957). If A_I is associated with *Isotricha* this would account for an occasional tendency in the various enzyme preparations to obtain a very high proportion of A_I to A_D.

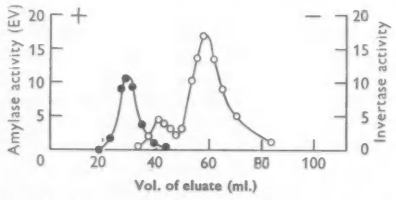

Fig. 3. Distribution of enzyme activity in eluate from column after zone electrophoresis of *Isotricha* extract. ○, Amylase activity; ●, invertase activity.

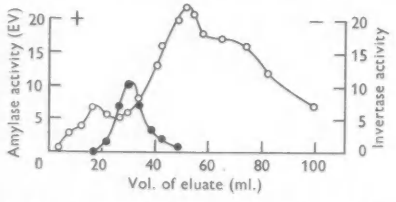

Fig. 4. Distribution of enzyme activity in eluate from column after zone electrophoresis of *Dasytricha* extract. ○, Amylase activity; ●, invertase activity.

The electrophoretic distribution of amylase activity due to *Dasytricha* (Fig. 4) shows a small peak of very high mobility (A_P) and a peak of fast-moving amylse (A_D) overlapped by invertase, with pronounced trailing. There is little doubt that the high value of EV still present at the end of elution of the column is indicative of adsorption, and it is possible that a small peak of slow-moving A_I is superimposed on this tail. No adsorption of the unfractionated *Dasytricha* enzyme extract during trial elution through the column was observed.

Electrophoretic mobility of enzymes. Unless the column conditions are standardized it is impracticable to determine absolute mobilities by measurements of migration. It is possible, however, to compare the unknown mobilities with that of a standard material, and the ovalbumin preparation was used for this purpose.

A comparative zone-electrophoretic fractionation of holotrich enzyme extract and ovalbumin in phosphate buffer, pH 7·0, I 0·05, at 20° is shown in Fig. 5. Boundary-electrophoresis determinations had to be made at I 0·1. In a corresponding fractionation on the column in phosphate buffer, pH 7·0, I 0·1, at 10° poorer separation was evident and also a change in the relative mobilities of the peaks.

The estimated mobilities of ovalbumin and conalbumin in boundary electrophoresis experiments made by Dr E. I. MacDougall were $5{\cdot}50 \times 10^{-5}$

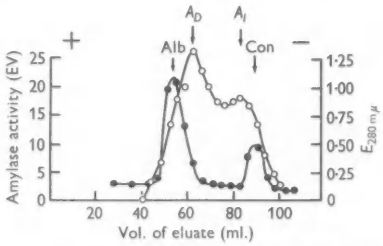

Fig. 5. Column-electrophoresis eluate of mixed holotrich extract and ovalbumin preparation. Phosphate buffer, pH 7, I 0·05, at 20°. ○, Amylase activity; ●, optical density at 280 mμ. (Alb. = albumin; Con. = conalbumin).

and $1{\cdot}48 \times 10^{-5}$ cm.^{2} v^{-1} sec.$^{-1}$ respectively in phosphate buffer, pH 6·94, I 0·1, at 0·6°. The holotrich enzyme extract under the same conditions gave two peaks $4{\cdot}23 \times 10^{-5}$ and $3{\cdot}31 \times 10^{-5}$ cm.^{2} v^{-1} sec.$^{-1}$. Assuming a proportional increase in the mobilities of the various components with temperature it was inferred that the mobilities of A_D and A_I are about $5{\cdot}4 \times 10^{-5}$ and $1{\cdot}3 \times 10^{-5}$ cm.^{2} v^{-1} sec. respectively in phosphate buffer, pH 7·0, I 0·1, at 0·6°.

Isoelectric points. A series of zone-electrophoretic separations of a mixture of holotrich extract and ovalbumin preparation were carried out at pH 7·00, 5·96, 5·04 (phosphate buffer, I 0·05) and pH 4·24 (acetate buffer, I 0·05). The eluate fractions were tested for amylase and invertase activity and for the presence of ovalbumin and the position of the zones located. The difference between the hold-up volume of the column and the elution volume of a peak was then assumed to be proportional to the electrophoretic mobility of the migrating substance under the particular column conditions. In order to eliminate approximately the different field conditions and electroendosmotic flow resulting from each buffer change, the mobilities of the enzymes were intrapolated from established values for the mobility of ovalbumin and conalbumin under free solution conditions. These electrophoretic mobilities are unlikely to be the same as under the conditions during electrophoresis on a packed-starch column, and will have no precise quantitative significance, but are valid for determining the conditions for zero mobility. The change in mobility with pH is shown in Fig. 6. No amylase activity was detected at pH 4·24. The isoelectric points of invertase and A_I were estimated as 4·15 and 5·40 respectively.

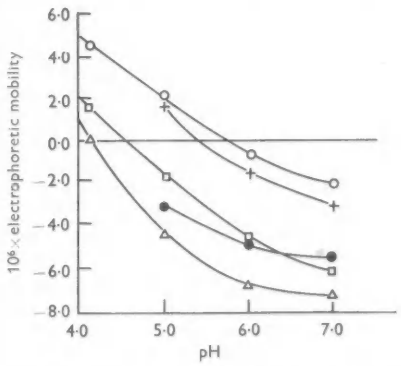

Fig. 6. Change in electrophoretic mobility with pH. See text. ○, Conalbumin; □, ovalbumin; +, amylase A_I; ●, amylase A_D; △, invertase.

Amylolytic properties of the amylases A_D and A_I

The reaction of these enzymes with amylose proceeded until the apparent conversion into maltose was 88%, and thereafter amylase A_D continued slowly to hydrolyse maltose and maltotriose. The electrophoretic separation of some of the enzymes in an extract of *Dasytricha* is shown in Fig. 7. It can be seen that maltase activity has not been separated from amylase during electrophoresis of an extract of *Dasytricha*. In extracts of *Dasytricha* and *Isotricha* of equal amylase activity it was also shown that the maltase activity of the *Dasytricha* extract was 20 times that of the *Isotricha* and that the *Dasytricha* extract was 6 times more active towards maltotriose. Thus it can be expected that the amylase A_D will hydrolyse amylose completely to glucose, whereas the action of A_I is arrested when amylose has been converted into maltose and maltotriose. Counts of the organisms present in the *Isotricha* and *Dasytricha* preparations are given in Table 2. The low maltase activity apparent in the *Isotricha* extract may be due to the presence of *Dasytricha*, although the activity seemed higher after hydrolysis for 24 hr.

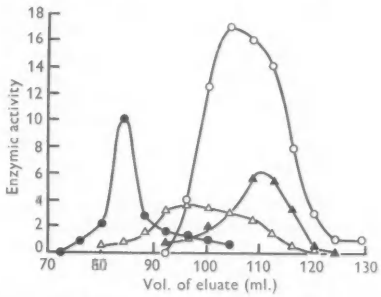

Fig. 7. Distribution of enzymic activity in column-electrophoresis eluate of *Dasytricha* extract. ●, Invertase; △, cellobiase; ○, amylase; ▲, maltase.

Table 2. *Count of* Isotricha *and* Dasytricha *present in purified preparations*

Protozoa were suspended in a 1:1 mixture of phosphate–acetate buffer and glycerol. A 1 ml. sample was placed in a counting cell and the contents of 25–30 squares were counted.

Preparation	Number of cells	
	Isotricha	*Dasytricha*
Isotricha	243	4
	242	3
	127	3
Dasytricha	6	736
	4	740
	2	583

than would be expected in relation to the proportion of organisms present.

The optimum temperature for the action of A_D and A_I on amylose was 55°. The optimum pH of action of A_I was 4·8. The activity curve for A_D showed two peaks, at pH 5·0 and 6·0 respectively (Fig. 8). Other amylases which show two maxima in their activity–pH curves are soya-bean α-amylase, malt α-amylase and pig pancreatic α-amylase.

It was of interest to know how the amylase activity per cell of *Dasytricha* compared with that of the *Isotricha*. For this purpose the holotrichs were separated in the usual manner, suspended in buffer, and divided into three portions. One portion was mixed with an equal volume of glycerol, and counts of the Protozoa were made in a 1 ml. counting chamber. The number of Protozoa in 25 squares taken at random from a possible 200 were counted. Another portion of the Protozoa in suspension were burst by freezing and thawing, and, since this procedure is not completely effective for the *Dasytricha*, the suspension was also ground in a glass tissue-crusher. The enzymic activities in the filtrates were then determined. The third portion of each suspension was dried in an oven at 100° overnight. Thus the amylase activity and dry weight of a given number of Protozoa were known. It was found that a single *Isotricha* contained 10·5 times the amylase activity of *Dasytricha*. Since by calculation an *Isotricha* is on average 9·1 times heavier than a *Dasytricha*, it follows that the amylase activity/unit weight is almost identical for the two species.

Action of extracts of Dasytricha *and* Isotricha *on disaccharides and polysaccharides*

The results for cellobiose and maltose are shown in Fig. 9. *Dasytricha* enzyme shows a far greater activity towards cellobiose than does the *Isotricha* extract. This compares with the result of Gutierrez (1955), who found that washed suspensions of *Isotricha* did not ferment cellobiose, whereas *Dasytricha* fermented cellobiose at a rate comparable with that for glucose. Dr G. Halliwell has tested a similar extract by his method for determining cellulase and found no attack on cellulose powder or hydrocellulose (Halliwell, 1957).

The results for maltose and maltotriose have already been discussed. Although the *Dasytricha* can hydrolyse maltose, it is not converted into storage polysaccharide. This is in contrast with the other hydrolysable carbohydrates sucrose, raffinose, inulin and bacterial levan, which can all be rapidly converted into storage polysaccharide by the holotrichs (Masson & Oxford, 1951).

The hydrolysis of sucrose and grass fructan by the holotrich extracts was measured by following the increase in reducing power of standard activity digests. For equal weights of dried Protozoa, the activity of the *Dasytricha* towards sucrose is 1·2 times that of the *Isotricha*. The corresponding value for grass fructan is 1·6.

Phosphorylase activity of holotrich Protozoa

The presence of amylase in the extracts of holotrich Protozoa has the effect of masking the system responsible for the synthesis of polysaccharide firstly by destroying the 'primer' necessary before synthesis can commence, and secondly by hydrolysing any polysaccharide that is synthesized. A search was therefore made for an inhibitor which

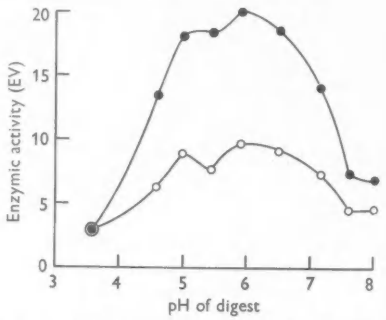

Fig. 8. Effect of pH on amylase A_D activity. ○, Incubated at 35° for 1 hr.; ●, incubated at 35° for 3 hr.

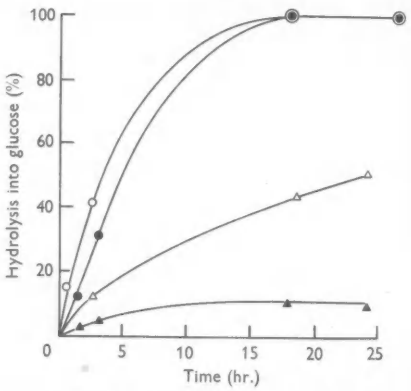

Fig. 9. Comparative action of extracts of *Dasytricha* and *Isotricha* on cellobiose and on maltose. ○, Extract from *Dasytricha* cells (3·4 mg. dry wt.) acting on cellobiose; ●, a similar extract acting on maltose; △, extract from *Isotricha* cells (7·1 mg. dry wt.) acting on cellobiose; ▲, a similar extract acting on maltose. Digests (6 ml.) contained 0·2 M-citrate buffer, pH 5·5, (1 ml.) and were incubated at 35°.

would selectively inactivate the amylase. Mercuric chloride was successfully used by Bailey, Thomas & Whelan (1951), who chose a concentration of $1{\cdot}54 \times 10^{-8}$ moles/unit of phosphorylase to inactivate the small amount of α-amylase which is associated with potato phosphorylase. It was not found possible completely to inactivate holotrich α-amylase by this method unless a concentration of mercuric chloride known to inactivate phosphorylase was chosen.

Wohl & Glimm (1910) reported that α-amylase is inhibited by 15% glucose. The use of this method for inactivating holotrich amylase was tested as follows. Various amounts of salivary α-amylase were added to potato phosphorylase in standard activity digests containing glucose 1-phosphate and starch 'primer'. After the amount of amylase required to inhibit phosphorylase had been determined, the effect of adding 15% glucose to the digests was tested. Synthesis, as measured by the liberation of inorganic phosphate, occurred in the tube containing phosphorylase only. No phosphate was liberated in the presence of α-amylase, irrespective of the presence of glucose, and furthermore glucose itself almost completely inhibited phosphorylase action. A similar test carried out with maltose gave the same result. In digests containing 15% of maltotriose, however, no inhibition of phosphorylase was caused either by the maltotriose or by the α-amylase.

Finally, a portion of freeze-dried holotrich extract (20 mg., 1 ml.) was incubated with glucose 1-phosphate (0·1 M) and maltotriose (500 mg.). Portions (0·3 ml.) were tested for free phosphate by Allen's method (1940). Equilibrium between glucose 1-phosphate and inorganic phosphate was reached in 3 hr. at 35°.

The presence of a phosphatase in the holotrich extract caused some liberation of inorganic phosphate in control digests containing no maltotriose, and this was prevented by the addition of 10^{-4} M-ammonium molybdate. Molybdate also inhibited the phosphoglucomutase action of holotrich extract.

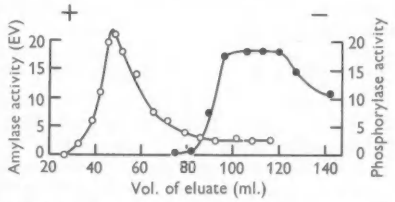

Fig. 10. Distribution of enzyme activity in column-electrophoresis eluate of *Dasytricha* extract (0·1 M-citrate buffer, pH 6·7). ○, Amylase activity; ●, phosphorylase activity.

Phosphorolytic degradation of amylose

A small amount of glucose 1-phosphate was found when a holotrich extract was allowed to react with amylose and phosphate buffer in the presence of ammonium molybdate and mercuric chloride. The percentage conversion into glucose 1-phosphate was very low (2·2%), since phosphorylase cannot degrade maltose and maltotriose, which are the main products of the amylolytic degradation of amylose.

Electrophoretic separation of phosphorylase and amylase

The possibility of separating amylase from phosphorylase in the holotrich extracts by zone electrophoresis on starch columns was investigated. Washed preparations of *Isotricha* were extracted in 0·1 M-citrate buffer (pH 6·7) and freeze-dried. A portion (46 mg.) of this extract was dissolved in water (1·5 ml.), centrifuged and eluted through the starch column at 20 ml./hr. with 0·1 M-citrate buffer (pH 6·7). The amylase distribution was symmetrical, whereas the phosphorylase was slightly retarded and exhibited adsorption tailing.

The distribution of amylase and phosphorylase activity after electrophoresis on the same column is shown in Fig. 10. The freeze-dried extract (334 mg.) was dissolved in water (2 ml.) and dialysed against water with frequent changes for 2 hr. until the salt concentration was reduced to 0·1 M. After centrifuging, the solution was applied to the column and electrophoresis carried out at 18° for 42 hr. under a p.d. of 258 v and column current 40 mA. The amylase peak was well-defined and separated from the phosphorylase activity, which was widely spread out. A preparation of potato phosphorylase, free from amylase, when subjected to zone electrophoresis under the same conditions gave a sharply defined narrow peak.

Conversion of amylose into branched polysaccharide

Results already presented have shown that the storage polysaccharide of the holotrich Protozoa is amylopectin, and there is no evidence for the presence of any linear polysaccharide. The holotrichs must therefore have a branching enzyme similar to the Q-enzyme of potato, which converts all the linear polysaccharide synthesized by phosphorylase into amylopectin. The test for this branching enzyme was complicated by the presence of amylase, which interfered by hydrolysing both amylose and amylopectin. Amylose was incubated with holotrich extract until the digest became achroic, then a qualitative test was made for isomaltose. It would be expected if the extract contained a branching factor that the amylopectin formed would be broken down by amylase to give

glucose, maltose and isomaltose. Glucose was removed by passing the digest through a charcoal–Celite column, and the disaccharides were eluted with aqueous 25 % (v/v) ethanol, and concentrated to dryness. Further separation of the disaccharides was achieved by zone electrophoresis on paper in borate buffer (pH 8·7), in an apparatus similar to that described by Latner (1952). A p.d. of 400 v was applied overnight, during which time the current rose from 9·5 to 11·5 mA. The paper was coloured with benzidine–trichloroacetic acid reagent (Bacon & Edelman, 1951), and the disaccharide fraction was found to contain both maltose and isomaltose. Isomaltose showed a characteristic pink colour after spraying with benzidine. A mixture of the disaccharides with isomaltose ran as maltose and isomaltose only. The distances moved (cm.) were: maltose 6·5, isomaltose 15·5, glucose 25·5. The same results were obtained with a synthetic amylose, having a chain-length of 55 glucose units, used as substrate in the original digest.

DISCUSSION

The work described in this paper has confirmed the results of Forsyth & Hirst (1953), who identified the storage polysaccharide of the holotrich ciliates as amylopectin. This has been achieved by the use of R-enzyme, which has the property of hydrolysing α-1:6-glucosidic linkages, thus allowing an examination of the inner chains of branched molecules to be carried out. When this enzyme is allowed to act on waxy-maize amylopectin, the intensity of the iodine stain increases to 165 % of its initial value, and the increase is accompanied by a slight shift of the absorption peak towards the red. At the same time the β-amylolysis limit of the amylopectin is raised from 51·5 to 100 %. On the other hand, glycogen treated with R-enzyme is not hydrolysed to any greater extent than glycogen by either α- or β-amylase, nor does the presence of active R-enzyme influence the degree of hydrolysis by crystalline β-amylase. The glycogen molecule is much more compact than amylopectin, the basal chains being shorter and the branch linkages nearer together, and it has been suggested (Peat *et al.* 1956) that this high degree of ramification renders the branch linkages of glycogen inaccessible to R-enzyme. A means of separating these linkages is provided by α-amylolytic degradation of glycogen, and this treatment does in fact render the branch linkages susceptible to R-enzyme action. The results for holotrich starch have shown that R-enzyme can increase the iodine absorption value of this starch, and the resulting starch treated with R-enzyme is completely hydrolysed to maltose by α-amylase. Further, the β-amylolysis limit of 51·5 % conversion into maltose is raised to 101 % when R-enzyme and β-amylase act simultaneously on the starch. This proves that holotrich starch is a typical amylopectin.

The results have also confirmed that the starch may be synthesized by the phosphorolytic mechanism common to both the plant and animal kingdoms. Other workers (Masson & Oxford, 1951) have determined which sugars may be utilized by the holotrichs for starch synthesis *in vivo*. The enzymes required for the hydrolysis of these carbohydrates to give glucose and fructose have all been detected in holotrich extract, and their activity estimated. Sugden & Oxford (1952) have reported that glucose 1-phosphate, when added to holotrich Protozoa in a suitable buffer, did not prolong their life, whereas the addition of cellobiose, fructose, glucose, sucrose, raffinose, inulin or bacterial levan extended the life of the culture. It is likely that glucose 1-phosphate is barred from permeating through the cell wall while the free sugars enter the cell, where they are hydrolysed to glucose and fructose by the appropriate enzyme and are then phosphorylated by hexokinase.

Maltose is the only disaccharide which can be hydrolysed to glucose without subsequent storage of starch. It is true that the maltase activity determined in holotrich extract is of a low order, yet it is comparable with cellobiase activity, and the holotrichs can utilize cellobiose for storage of starch. An explanation might be that maltose is utilized preferentially by some other enzyme system so far undetected.

Table 3. *Summary of properties of α-amylases*

The table is taken from Fischer & de Montmollin (1951*a*, *b*), with the addition of data for A_D and A_I obtained in the present work. Abbreviations: G, glucose; M, maltose; T, maltotriose.

	Malt	*Aspergillus oryzae*	A_D	A_I	Rumen *Streptococcus bovis*	*Bacillus subtilis*	Pig pancreas	Human saliva
Optimum pH	4·1, 5·4	5·5–5·9	5·0, 6·0	4·8	5·5–6·5	5·3–6·8	6·9	6·9
Activation								
By Ca^{2+} ions	+	−	−	−	−	−	−	−
By Cl^- ions	−	−	−	−	−	+	+	+
Isoelectric point	5·6	4·2	<5	5·4	.	.	5·3	5·3
Product of action on amylose	G, M	G, M	G, M, T	M, T	M, T	G, M	G, M	G, M, T

The zone-electrophoretic fractionation of aqueous extracts of holotrichs has overcome some of the difficulties in a complete physical separation of the *Isotricha* and *Dasytricha* and has permitted the characterization of the α-amylases specific to each organism. The overlapping of the two activity curves in varying proportions would otherwise have obliterated the characteristic pH optima given by the two enzymes.

It is of interest to compare the properties of the holotrich amylases with those of other amylases of plant and animal origin. For this purpose our results have been combined with those of Fischer & de Montmollin (1951*a*, *b*) in Table 3. A_D and A_I may be grouped with the amylases of *Aspergillus oryzae* and the rumen *Streptococcus* (Hobson & Macpherson, 1952), all of which are independent of the presence of Cl^- or Ca^{2+} ions.

An attempt has been made to fractionate these enzymes by absorption chromatography on hydroxyapatite columns eluted with phosphate buffer of increasing concentration (Tiselius, Hjertén & Levin, 1956; Semenza, 1957). Differences in the elution curves and in the activity–pH curves of the eluted peaks of single organism preparations are evident although the enzymes have not been completely resolved. The zone-electrophoretic fractionations so far appear to have been more satisfactory.

SUMMARY

1. The action of the debranching enzyme R-enzyme, α-amylase and β-amylase on starch stored by holotrich ciliates from the sheep rumen has verified that this starch is amylopectin.

2. A comparison of various enzyme activities present in aqueous extracts of purified preparations of the single organisms *Isotricha* and *Dasytricha* has been made.

3. In the presence of 15% maltotriose, holotrich α-amylase is inhibited but phosphorylase activity is unaffected.

4. Holotrich starch is synthesized by the phosphorolytic mechanism common to plants and animals.

5. Two α-amylases specific to *Isotricha* and *Dasytricha* have been resolved by zone electrophoresis of holotrich extracts on starch columns.

6. These amylases differ in optimum pH, electrophoretic mobility and isoelectric points. They are not activated by Ca^{2+} or Cl^- ions.

7. Zone-electrophoretic separation of phosphorylase and amylase was unsuccessful owing to adsorption of phosphorylase on the column.

We wish to thank Dr E. I. McDougall for boundary-electrophoresis determinations, and Miss E. Clark and Mr A. Dawson and Mr W. Smith for technical assistance.

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Studies on the Skeletal Tissues

5. THE INFLUENCE OF AGE UPON THE DEGREE OF CALCIFICATION AND THE INCORPORATION OF ^{32}P IN BONE*

By S. M. WEIDMANN
The Biological Research Unit, The Dental School and Hospital, University of Leeds
AND H. J. ROGERS
National Institute for Medical Research, Mill Hill, London, N.W. 7

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Earlier work has established that as animals grow the cortical part of the long bones becomes more calcified (for example, Strobino & Farr, 1949; Rogers, Weidmann & Parkinson, 1952; Tarján & Szöke, 1957) and the bones incorporate decreasing amounts of administered isotopic tracers (for example, Falkenheim, 1942–3; Bonner, 1948). It has also been made apparent that the examination of samples of powdered whole cortical bone from young or adult animals gives only an average value for the tissue which is made up of parts with widely differing properties (Amprino & Engström, 1952; Engfeldt, Engström & Zetterström, 1952; Rogers, Weidmann & Jones, 1953; Tomlin, Henry & Kon, 1953; Weidmann, 1956). Since these differences are expressed at a microscopic level, i.e. each Haversian system seems likely to have its own degree of calcification and rate of incorporation of tracer, logically each should be examined separately. Radioautographic and X-ray studies such as those by Leblond, Wilkinson, Bélanger & Robichon (1950), Engfeldt *et al.* (1952) and Tomlin *et al.* (1953) have provided a semi-quantitative picture of the variations at the microscopic level in calcification and incorporation of ^{32}P in both adult and growing animals. In the present work an attempt has been made to obtain precise measurements on restricted regions and types of bone and thus to compromise between methods which have exactly measured the properties of whole bone samples and those which have assessed incorporation of ^{32}P at the microscopic level by radioautographic techniques. The two species of animals, cats and rabbits, were chosen because earlier work (Rogers *et al.* 1953) had shown certain differences existed between them and because the young animals of both species were large enough to allow the proper handling and cleaning of the bones.

METHODS

Owing to the large number of experiments involved and to local conditions, animals of various litters and breeds had to be used. In order to minimize complications due to variations between the growth rate of different breeds, each age group was constituted of animals which had shown similar rates of growth. The groups of adult animals of both species were selected over a considerable period of time and consisted of apparently healthy animals of weight greater than 2 kg. The ages within this group are consequently likely to vary rather widely. However, as will be seen from the recorded results, this does not seem to lead to greater variability in the bone characteristics than within the more exactly defined groups. The standard of error of the means of the results from the adult animals are no greater than those from the other groups.

The methods of dissecting and cleaning bones have been previously described (Weidmann & Rogers, 1950) and have proved satisfactory for the removal of bone marrow from the small bones of young animals. The central parts of the shaft cortex of the femoral and tibial bones were used for the investigation of cortical bone, and the metaphyseal trabeculae from both bones for cancellous tissue. In one-day-old animals the entire shaft is still highly vascularized and has a trabecular appearance, so that no complete distinction is possible between cortical and cancellous bones. From the age of 5 days onwards the metaphysis and diaphysis could be distinguished and were separated. In very young animals, however, particularly in young cats, the centre part of the medullary shaft was still covered with trabecular spicules, which could not be removed and so were included along with the samples of cortical bone. In order to obtain adequate amounts for the various estimations, bone-powder samples from femur and tibia had to be pooled. Calcium, phosphorus and nitrogen estimations, together with radioactivity measurements, were made as previously described (Weidmann & Rogers, 1950; Rogers *et al.* 1953). The activities of the subperiostal and subendosteal layers were estimated by the paraffin-protection method described by Weidmann (1956). The administration of ^{32}P was adjusted to body weight and 33 μc/kg. was injected intravenously in rabbits or intraperitoneally in cats. The animals were always killed for the measurement of radioactivity in the bones 4 hr. after injection of the isotope. The specific activity was expressed, as in previous work (Rogers *et al.* 1953), by the fraction

$$\frac{(\text{Counts/min./mg. of P}) \times 10^6}{\text{Administered counts/min./kg. body wt.}}.$$

* Part 4: Weidmann, S. M. (1956).

Table 1. *Chemical composition of cortical bones in growing rabbits*

Results are mean values ±S.E.M. and are given as a percentage of dry, defatted bone. The Ca/P ratios are means of ratios and not ratios of means.

Age (days)	Wt. (g.) Mean	Wt. (g.) Range	No. of animals	Ca (%)	P (%)	Ca/P	N (%)	Ca/N
Embryo	—	—	2	25·4±0·28	12·0±0·14	2·10±0·05	—	—
1	54	31–77	26	25·2±0·14	11·6±0·08	2·17±0·01	3·93±0·04	6·36±0·09
5	89	61–123	16	24·9±0·21	11·2±0·05	2·22±0·02	4·09±0·06	5·96±0·08
9	145	135–175	12	25·4±0·21	11·6±0·19	2·19±0·04	4·12±0·09	6·20±0·16
14	245	210–290	8	25·7±0·30	12·2±0·13	2·12±0·13	4·17±0·06	6·21±0·13
28	447	342–700	11	26·5±0·02	12·4±0·08	2·12±0·02	3·65±0·06	7·28±0·16
84	794	700–1040	14	27·8±0·23	12·8±0·11	2·18±0·02	3·36±0·05	8·25±0·16
Adults	3000	2250–4000	46	28·6±0·15	12·7±0·06	2·27±0·01	3·21±0·03	8·87±0·10

Table 2. *Chemical composition of cortical bones in growing cats*

Results represent mean values ±S.E.M. and are given as a percentage of dry, defatted bone. The Ca/P and Ca/N ratios are means of ratios and not ratios of means.

Age (days)	Wt. (g.) Mean	Wt. (g.) Range	No. of animals	Ca (%)	P (%)	Ca/P	N (%)	Ca/N
1	90	55–114	8	25·0±0·32	11·4±0·22	2·20±0·03	4·40±0·10	5·70±0·11
5–6	115	95–145	13	24·5±0·24	11·4±0·14	2·17±0·02	4·62±0·02	5·35±0·07
7–9	200	97–236	14	24·5±0·10	11·5±0·09	2·15±0·02	4·87±0·08	5·06±0·09
21	246	207–290	6	24·1±0·24	11·5±0·14	2·10±0·02	4·87±0·08	4·95±0·12
42	450	290–598	13	25·2±0·22	11·4±0·11	2·22±0·02	4·60±0·05	5·49±0·08
84	690	585–780	6	25·2±0·39	11·4±0·08	2·18±0·03	4·66±0·06	5·35±0·17
180	1450	1150–2000	9	26·3±0·13	11·8±0·08	2·22±0·01	4·34±0·07	6·01±0·15
Adults	3000	2100–3600	27	27·4±0·14	12·2±0·06	2·24±0·02	4·01±0·06	6·97±0·12

RESULTS

Variations in chemical composition

Cortical bone. The calcium content of the cortical bone of both the rabbit and cat increases during growth, but, since the phosphate concentration also increases, the calcium/phosphorus ratio varies only slightly. The maximum limits of variation are from 2·10 to 2·27 (Tables 1 and 2). The total increases in both calcium and phosphorus concentrations are greater in the rabbit than in the cat. These increases, however, do not start in the rabbit until it is 1 week old and in the cat until it is 3 weeks old. Until this time, in the cat, the calcium concentration decreases slightly, whereas the phosphorus remains constant and the nitrogen increases slightly; consequently the calcium/nitrogen ratio decreases considerably, and the calcium/phosphorus ratio also decreases slightly. There is possibly a similar initial decrease in the calcification of the cortical bones of young rabbits, but it is much smaller.

Cancellous bone. Much less change is observed in the calcium and nitrogen concentrations of the cancellous bones of the rabbit (Table 3). Thus the degree of calcification of this type of bone, as measured by the calcium/nitrogen ratio, does not change significantly throughout the life of the animals. In the cat (Table 4) the degree of calcification falls quite markedly throughout growth.

Variations in the incorporation of ^{32}P during growth

The specific activities of samples of whole cortical and cancellous bones show the expected marked decrease during growth in both the rabbit and the cat, but there are differences between the two species of animal (Tables 5 and 6).

Cortical bone. The specific activity of phosphorus in the cortical bone of the rabbit (Table 5) is greatest in newly born animals and falls continuously during growth. In the cat (Table 6) the specific activity is highest in the 7- to 9-day-old group of animals, exceeding that of newly born kittens. After this maximum has been reached, a fall is observed with increasing age; the gradient of decline is not initially as steep as in the rabbit.

Cancellous bone. Specific activity in the cancellous bones of both species is greater throughout life than in the cortex. It rises in the cancellous bones of the cat in the first weeks of life, as in the cortical bones, but the increase is somewhat greater. This phenomenon of a maximum in the degree of incorporation into both types of bone during life seems to be characteristic of the cat.

Table 3. *Chemical composition of cancellous bones in growing rabbits*

Results represent mean values ±S.E.M. and are given as a percentage of dry defatted bone. The Ca/N ratios are means of ratios and not ratios of means.

Age (days)	Wt. (g.) Mean	Wt. (g.) Range	No. of animals	Ca (%)	P (%)	Ca/P	N (%)	Ca/N
1	54	31–77	26	—	—	—	—	—
5	89	61–123	16	24·8±0·25	11·6±0·08	2·13±0·02	4·34±0·15	6·00±0·22
9	145	135–175	12	25·2±0·18	11·7±0·19	2·19±0·12	4·06±0·13	6·31±0·17
14	245	210–290	8	25·0±0·29	11·9±0·11	2·08±0·05	4·50±0·11	5·58±0·18
28	447	342–700	11	25·4±0·30	11·8±0·10	2·12±0·02	3·87±0·10	6·63±0·23
84	794	700–1040	14	25·5±0·17	11·6±0·10	2·19±0·02	4·41±0·08	5·75±0·13
Adults	3000	2250–4000	46	26·2±0·15	11·4±0·06	2·29±0·01	4·37±0·05	6·00±0·09

Table 4. *Chemical composition of cancellous bones in growing cats*

Results represent mean values ±S.E.M. The Ca/P and Ca/N results are means of ratios and not ratios of means. The results are given in terms of dry, defatted bone.

Age (days)	Wt. (g.) Mean	Wt. (g.) Range	No. of animals	Ca (%)	P (%)	Ca/P	N (%)	Ca/N
1	90	55–114	8	26·8±0·11	12·0±0·03	2·23±0·03	4·09±0·05	6·56±0·06
5–6	115	95–145	13	25·0±0·30	11·8±0·18	2·12±0·03	4·05±0·10	6·40±0·13
7–9	200	97–236	14	25·6±0·26	11·9±0·07	2·16±0·02	4·32±0·06	6·12±0·08
21	246	207–290	6	25·0±0·31	11·8±0·22	2·12±0·22	4·44±0·06	5·65±0·14
42	450	280–598	13	24·2±0·16	11·1±0·09	2·19±0·02	4·71±0·05	5·13±0·04
84	690	585–780	6	24·5±0·33	11·3±0·08	2·17±0·02	4·93±0·10	4·97±0·05
180	1450	1150–2000	9	24·6±0·27	11·3±0·23	2·17±0·01	4·94±0·09	4·91±0·11
Adults	3000	2100–3600	27	25·2±0·15	11·2±0·08	2·27±0·02	4·91±0·06	5·31±0·09

Table 5. *Specific activity of inorganic phosphorus in bones of growing rabbits 4 hr. after the injection of 33 μc/kg. body wt. of carrier-free ^{32}P as $H_3{}^{32}PO_4$*

Results represent mean values ±S.E.M. For definition of specific activity, see Methods.

Age (days)	Wt. (g.) Mean	Wt. (g.) Range	No. of animals	Specific activity Cortical bone	Specific activity Cancellous bone
1	54	31–77	22	682±46	—
5	97	72–123	11	602±50	1620±139
9	147	135–175	12	525±43	1044±95
14	239	210–290	10	484±34	1020±96
28	447	342–700	10	216±43	854±121
84	794	700–1040	9	115±19	735±101
Adults	2960	2250–4200	28	48±5	332±37

Table 6. *Specific activity of inorganic phosphorus in bones of growing cats 4 hr. after the injection of 33 μc/kg. body wt. of carrier-free ^{32}P as $H_3{}^{32}PO_4$*

Results represent mean values ±S.E.M. For definition of specific activity, see Methods.

Age (days)	Wt. (g.) Mean	Wt. (g.) Range	No. of animals	Specific activity Cortical bone	Specific activity Cancellous bone
1	90	55–114	8	396±44	660±57
5–6	115	95–145	10	389±46	507±71
7–9	194	97–236	17	618±42	1204±101
21	247	228–268	4	553±58	1403±245
42	458	280–598	17	330±23	1390±117
84	692	585–780	9	206±25	886±112
180	1420	1150–1700	11	81±9	627±121
Adults	2940	2100–3500	15	19±3	273±142

Incorporation of ^{32}P in various layers of cortical bone during growth

The salient points of the work of Tomlin *et al.* (1953) are confirmed by the present results, but some findings are different, as might be expected from the different experimental approach. A single administration of ^{32}P was used in this work and the animals were killed 4 hr. later, as compared with the prolonged intake of ^{45}Ca used by Tomlin *et al.* (1953); hence the present results are likely to stress rapid exchange of the phosphate instead of reflecting deposition over a long period of time, which was the interest of the other authors. Shaft bones from at least three animals of each age group were examined by the paraffin-protection method (Weidmann, 1956) and the specific activities of phosphorus determined in the subperiosteal and subendosteal layers, together with the average activity of the shaft.

In younger rabbits (Table 7) the activity of the phosphorus in the subperiosteal layer is greater than in the subendosteal layer. The subperiosteal layer remains comparatively active in all periods of growth and decreases only when the animal reaches maturity. In the subendosteal layer, however, the activity increases gradually during growth. When the animal reaches about one-third of its full development (84 days) tracer incorporation occurs at about equal rates on both surfaces of the tubular shaft; in maturity the incorporation is some five to six times as great on the surface next to the marrow. A comparison of the specific activities of the two surface layers with those of the average activities in growing and mature shafts shows that the whole bone is relatively active during growth, but that, in the fully formed shaft, the activity is almost completely restricted to the subperiosteal and subendosteal layers of the bone, and particularly to the latter.

In the growing cat examination of the regional activities of the developing diaphysis shows a similar picture to that in the rabbit (Table 8). During the first weeks of post-natal life the specific activity in the periosteal region is much higher than that next to the endosteum. The subperiosteal activity decreases rapidly and the difference between the developing and mature shaft is very large in this region. There is a gradual increase in the specific activity of the endosteal region during growth, reaching that of the periosteal region at the age of 84 days and surpassing it in the later phases of life. The overall picture presented by both species of animal is the same.

DISCUSSION

One aspect of this work is to emphasize species difference and localized effects in the biochemical behaviour of the skeleton during growth. For

Table 7. *Specific activity of inorganic phosphorus in various layers of cortical bone of growing rabbits 4 hr. after the injection of* ^{32}P

Results represent mean values ±S.E.M. For the definition of specific activity, see Methods.

Age (days)	Wt. (g.) Mean	Wt. (g.) Range	No. of animals	Specific activity: Cortical bone	Specific activity: Subperiosteal layer	Specific activity: Subendosteal layer
5	97	84–110	3	829±14	1580±127	565±108
9	156	135–175	3	538±72	2248±371	520±41
14	229	210–263	4	438±63	1357±422	942±252
28	398	334–472	3	335±29	2262±182	771±6
84	889	819–939	3	118±32	1257±135	1557±376
Adults	2732	2450–3140	9	46±3	654±133	3910±666

Table 8. *Specific activity of inorganic phosphorus in various layers of cortical bone of growing cats 4 hr. after the injection of* ^{32}P

Results represent mean values ±S.E.M. For the definition of specific activity, see Methods.

Age (days)	Wt. (g.) Mean	Wt. (g.) Range	No. of animals	Specific activity: Cortical bone	Specific activity: Subperiosteal layers	Specific activity: Endosteal layers
9	191	174–213	4	738±103	3598±890	990±205
21	245	228–260	4	553±58	1849±413	683±52
42	535	445–598	7	412±34	2597±290	1138±145
84	668	585–780	6	233±60	1654±273	1308±137
180	1407	1170–1625	6	80±11	1900±202	3472±549
Adults	2813	2345–3470	6	29±6	477±70	2180±313

example, the cancellous trabeculae from the metaphyseal regions of cat bones, far from becoming more calcified during growth, actually show a marked decrease in the ratio of calcium to total nitrogen content as the animals get older, a change which continues almost throughout life. The cortex, too, of the long bones in these animals starts to increase in degree of calcification only after the animals have reached about 10 % of their adult weight. During the first two- or three-fold weight increase the degree of calcification declines, as indicated by a reduction of the calcium/nitrogen ratio from 5·70 to 4·95. This conclusion is unlikely to be upset by the inclusion of some trabeculae into the cortical samples from the very young cats since the amount of trabecular bone is decreasing rapidly during the first fortnight of life. This should mean that there is a decreasing contamination of the cortical bone, which would tend to emphasize an increase rather than a decrease in degree of calcification. In the rabbit, on the other hand, the cancellous bones remain approximately constant in composition throughout growth, whereas the cortex starts to increase in calcification as soon as the newly born animals' weight has increased rather more than 50 %, i.e. when it has achieved only 3 % of its adult weight. The division of bones into cortex and trabeculae, which has allowed adequate samples to be obtained throughout growth, is still very crude, and no doubt much more striking differences could be shown by examining more closely localized samples. Even from this work, however, it appears that during growth of the skeleton local species-specific alterations occur in the relative rates of deposition of salts and collagen, over 95 % of the nitrogen of adult bone being due to collagen (Rogers *et al.* 1952; Eastoe & Eastoe, 1954; Tarján & Szöke, 1957) in all species of animal so far examined. Tarján & Szöke (1957) have found that there is also a much larger proportion of the total nitrogen due to water-soluble compounds in bones of young rats. It still seems likely, however, that most of the alteration in total nitrogen of bones is due to changes in collagen-like substances, since the water-soluble substances of Tarján & Szöke (1957) have a large content of hydroxyproline (private communication) and may represent some form of soluble collagen.

Burns & Henderson (1935) drew attention to the decrease in calcium content of the cortical and trabecular bones which takes place during the growth of young kittens and suggested (Burns & Henderson, 1936), as had Bauer, Aub & Albright (1929), that the calcified trabeculae may form a reserve to be drawn upon during subsequent growth. If indeed the decrease both in quantity of trabecular bone and in the degree of calcification of those trabeculae that remain indicates that their salts are being used as an endogenous source for the growing skeleton (i.e. as a reserve), then the increase in incorporation of ^{32}P which occurs in week-old kittens in both cortical and trabecular bone may be a further reflexion of the mobilization and translocation of phosphate. It would be of great interest to study the influence of nutritional and hormonal status on the composition and metabolism of the cancellous bones of young kittens.

In an attempt to obtain more information about the localized metabolic differences the rapid uptake of ^{32}P has been examined in the surface layers under the periosteum and endosteum from the bones of the growing animals. The specific activities in these two layers show changes which are large when compared with those of the whole bone. In both species the subperiosteal layer declines in activity, whereas the subendosteal layer reaches its greatest activity in the mature animal. It seemed possible that the declining activity of the periosteal layer was reflecting the reduction in rate of deposition of bone salts which necessarily occurs as the circumferential growth rate of bone declines. This interpretation, however, cannot be accepted without reserve since Leblond *et al.* (1950) also found a periosteal zone of active ^{32}P deposition in the shaft of long bones by radioautographic technique at as short a time interval as 5 min. after intravenous injection of the isotope into rats. It would seem that deposition is not likely to play a significant part in defining this layer in a time interval of 5 min. and may not do so in the longer time of 4 hr. used here. Possibly some such factor as better blood supply or the easier penetration of ions into freshly deposited bone-salt crystals in very young animals may be involved (Neuman & Neuman, 1953). Leblond *et al.* (1950) also found that there is an endosteal layer of bone salt, which rapidly incorporates isotope, at the end regions of the shaft where it is expanding into the epiphysis. They refer to these regions as the funnels. This observation, however, is unlikely to help in interpreting the present results since only the middle region of the shaft has been used and the regions of the funnels are excluded. It is conceivable that the high value for the incorporation of phosphate into the endosteal layer of adult bone is due to a good regional blood supply; but, if so, it is also true, as Weidmann (1956) points out, that some other factor must intervene to prevent rapid equilibration of this layer with the blood phosphate in the cat. In this animal he showed that the maximum value for the endosteal layer is reached only 4 hr. after the injection of ^{32}P, whereas it is reached within 30 min. in the rabbit. In both species the specific activity of the serum phosphate reached a maximum in 30 min. despite the different routes

of administration of the isotope, which was given intravenously in the rabbit and intraperitoneally in the cat.

SUMMARY

1. The degree of calcification (calcium/nitrogen ratio) of cortical bones in rabbits increases from about the fifth day of life and in the cat from about the ninth day of life. From the birth of kittens until they are 9 days old there is a continuous reduction in the degree of calcification.

2. The calcification of the cancellous bone in rabbits remains approximately constant during the whole of growth, whereas in cats this bone becomes less calcified during growth.

3. The amount of ^{32}P incorporated during the 4 hr. following administration declines throughout the growth of rabbits in both cancellous and cortical bone. In cats there is a maximum incorporation in both types of bone at an age of about 14 days. This maximum is more marked in the cancellous bone.

4. In both cats and rabbits there is a very marked change during growth in the incorporation of ^{32}P in the subperiosteal and endosteal layers of bone. The incorporation of ^{32}P in the former layers declines throughout growth, whereas that in the latter layers reaches its maximum in the adult.

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Enzyme Systems in Marine Algae

2. TRANS-α-GLUCOSYLATION BY EXTRACTS OF *CLADOPHORA RUPESTRIS**

By W. A. M. DUNCAN† and D. J. MANNERS
Department of Chemistry, University of Edinburgh

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Duncan, Manners & Ross (1956) reported that unfractionated extracts of four species of marine algae (*Cladophora rupestris*, *Laminaria digitata*, *Rhodymenia palmata* and *Ulva lactuca*) showed hydrolytic activity towards a number of carbohydrates, including maltose. In dilute aqueous solution (0·04 %) this sugar was completely hydrolysed to glucose, whereas in concentrated solution (7–17 %) the synthesis of oligosaccharides was observed. By contrast, incubation of an algal extract with a concentrated solution of glucose did not result in oligosaccharide synthesis. The enzymic reaction therefore involves the transfer of α-glucosyl residues to glucosaccharides and not the enzymic polymerization of glucose; in this respect, the algal enzyme systems differ from that in *Aspergillus niger* (strain NRRL 330), which can synthesize disaccharides from glucose (Peat, Whelan & Hinson, 1955).

In the present paper the characterization of the oligosaccharides synthesized from maltose by an extract of *Cladophora rupestris* is described, and the acceptor specificity of the trans-α-glucosylase system is discussed.

* The paper by Duncan, Manners & Ross (1956) is regarded as Part I.

† Present address: Imperial Chemical Industries Ltd., Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire.

METHODS AND MATERIALS

Analytical methods

Chromatography. (*a*) *Qualitative.* Paper chromatography of free sugars was carried out as previously described with either *n*-butanol–pyridine–water–benzene (5:3:3:1, by vol.) (*A*) or ethyl acetate–pyridine–water (10:4:3, by vol.) (*B*) as solvent. The rate of movement of sugars is expressed relative to D-glucose (R_G values) (Duncan *et al.* 1956). The corresponding *N*-benzylglucosylamines were chromatographed by the method of Bayly & Bourne (1953).

(*b*) *Preparative.* Columns containing a 1:1 (w/w) mixture of activated charcoal (British Drug Houses Ltd.) and Celite no. 545 (Johns Manville Co., New York, U.S.A.) were prepared by the general methods of Whistler & Durso (1950), Whelan, Bailey & Roberts (1953) and Bacon (1954). Oligosaccharides were eluted with an increasing concentration of aqueous ethanol; fractions of 700 ml. were collected from the larger columns (70 or 79 cm. × 4·5 cm.), and fractions of 7 ml. from smaller columns.

Electrophoresis. Paper electrophoresis of free sugars was carried out in a water-cooled apparatus (Foster, 1952) with Whatman no. 1 paper in a 0·1N-borate buffer (pH 10·0), with a 1 hr. period for equilibration. The current (500V; 12·5 mA) was passed for 3·5 hr., the paper was air-dried and the sugars were located with an aniline oxalate–acetic acid spray reagent.

Periodate oxidation (with Mr F. B. Anderson). The oligosaccharides were oxidized with sodium metaperiodate in the presence of 0·05M-phosphate buffer (pH 8·0) as described by Hough & Perry (1956). The production of formaldehyde was determined with a phenylhydrazine–ferricyanide reagent (Hough, Powell & Woods, 1956). Under these conditions 1→4-linked oligosaccharides yield 1 mole of formaldehyde/glucose residue, whereas formaldehyde is not liberated from glucose residues which are joined by 1→6 linkages.

Estimation of degree of polymerization (*DP*). The sugars (about 5 mg.) were dissolved in water (10 ml.) and the reducing power of samples (2 ml.) was estimated with the iodometric Shaffer & Somogyi (1933) reagent as modified by Hanes & Cattle (1938). With the exception of M_0, which required 20 min. heating, a heating time of 60 min. was necessary for the full development of reducing power. The reducing powers were calculated as equivalents of maltose. Samples (2 ml.) were hydrolysed with sulphuric acid (36N; 0·12 ml.) at 100° for 2 hr., neutralized with sodium hydroxide (phenolphthalein), diluted to 5 ml. and the glucose content was determined.

Partial acid hydrolysis. The sugar (1%, w/v solution; 1 ml.) and sulphuric acid (0·5N; 0·6 ml.) were heated at 100° for 1 hr., neutralized, concentrated and analysed by paper chromatography. Alternatively, the sugar (1% solution; 5 ml.), iodine (30 mg.) and potassium hydroxide (1·0N; 0·2 ml.), mixed in this order, were shaken together, and after the solution had been kept for 30 min. a further portion of alkali (0·1 ml.) was added. After 15 hr. incubation at room temperature, a sample of the aldonic acid solution (1 ml.) was partly hydrolysed as described above.

Enzyme preparations

Extract of C. rupestris. Soluble protein was extracted from fresh minced algae as previously described (Duncan *et al.* 1956). The freeze-dried material contained 8·8% of nitrogen.

β-Amylase. A commercial preparation from barley was used (Liddle & Manners, 1957).

D-Enzyme. This enzyme was prepared from potato juice by the method of Peat, Whelan & Rees (1956).

EXPERIMENTAL AND RESULTS

Preparation and isolation of oligosaccharides

A digest was prepared containing recrystallized maltose (32·5 g.), 0·1M-acetate buffer (pH 5·0; 250 ml.), freeze-dried extract (1·25 g.) and toluene. After incubation at 35° for 16 days, the digest was heated to inactivate the enzyme and concentrated. Paper-chromatographic analysis showed the presence of glucose, maltose, and six additional oligosaccharides with R_G values (in solvent *A*) ranging from 0·38 to 0·03 (see Table 1). (R_G values are relative to D-glucose.)

To facilitate separation of the oligosaccharides, the concentrate was chromatographed on a charcoal–Celite column (79 cm. × 4·5 cm.) and the glucose and maltose were eluted with water and 4% (v/v) aqueous ethanol respectively. The oligosaccharides were then eluted with 30% ethanol and concentrated.

The resulting syrup was applied to a second column (70 cm. × 4·5 cm.) and the oligosaccharides were eluted with increasing concentrations of ethanol. Fractions (700 ml.) were collected, concentrated and examined by paper chromatography. None of the fractions became acid on concentration.

The oligosaccharides will be referred to as M_0, M_1, M_2, etc., in order of elution from the column; in a preliminary comment on the present work (Duncan *et al.* 1956) the two major sugars were named in order of paper-chromatographic mobility, so that M_1 and M_2 are now redesignated M_2 and M_1 respectively.

The following combined fractions were collected: *A*, glucose; *B*, maltose and M_0 (faint trace); *C*, maltose; *D*, maltose and M_1; *E*, M_1 (1·38 g.); *F*, M_1, M_2, and maltose (trace); *G*, M_2 and maltose (faint trace); *H*, mixed higher oligosaccharides.

Fraction *G* was chromatographed on a third column (65 cm. × 3 cm.), yielding pure M_2 (1·50 g.). Fraction *H* was applied to a fourth column (67 cm. × 3 cm.). Elution with 0–2 and 5% aqueous ethanol gave chromatographically pure glucose and M_0 (25 mg.) respectively. Elution with increasing concentrations of ethanol (6% → 18%) was continued, 7 ml. fractions being collected. The yields of the pure oligosaccharides, after freeze-drying or crystallization from ethanol, are given in Table 1. Mixed fractions were not analysed further, except that chromatography of a mixture

of M_2 and M_3 on Whatman no. 3 MM paper yielded pure M_3 (10 mg.).

A control digest containing maltose (8·1 g.) and 0·1 M-acetate buffer (pH 5·0; 63 ml.) was incubated at 35° for a similar period. After heating, it was concentrated, and applied to a charcoal–Celite column (21 cm. × 4·5 cm.) and developed with increasing concentrations of aqueous ethanol. No sugar other than maltose could be detected. The observed synthesis of oligosaccharides in the main digest is not therefore due to microbial contamination of the buffer and sugar solution. Further, the purity of the maltose used as substrate is confirmed.

Preliminary characterization of the oligosaccharides

The purified oligosaccharides, which were homogeneous by paper chromatography and electrophoresis, were reducing sugars which contained glucose and no other sugar.

The results in Table 1 indicate that M_2 and M_3 are trisaccharides and that M_4, M_5 and M_6 are tetrasaccharides. Further, since the degree of polymerization (DP) values of M_2 and M_5 are very close to whole numbers, these sugars are probably maltosaccharides (cf. Whelan *et al.* 1953).

N-Benzylglycosylamine derivatives of M_1, M_2, maltose and maltotriose were prepared; the R_G values were 0·29, 0·31, 0·57 and 0·31 respectively.

The four main oligosaccharides and the corresponding aldonic acids were partly hydrolysed with acid, and the products were tentatively identified by paper chromatography (Table 2).

Further evidence of the structure of M_1, M_2, M_4 and M_5 was obtained by determining the formaldehyde liberated during 'over-oxidation' with periodate (see Table 1). In a control experiment, maltose gave 1·8 moles/mole (theoretical, 2·0).

In addition to the above observations, molecular rotation and paper-chromatographic mobility [expressed as $\log R_G/(1-R_G)$] data support the suggestion that M_2 and M_5 are maltotriose and maltotetraose respectively. A comparison of the figures with those of maltose shows that a linear relationship exists with the DP of the sugar. This indicates that maltose, M_2 and M_5 belong to the same polymeric series of sugars (cf. Whelan *et al.* 1953). Further, the paper-chromatographic mobilities of M_1 and M_4 are intermediate between those of the maltosaccharides and the isomaltosaccharide series, suggesting that M_1 and M_4 may contain both α-1 → 4- and α-1 → 6-glucosidic linkages (cf. French & Wild, 1953). M_0, M_3 and M_6 have not been examined further.

Characterization of M_1 *as panose*

M_1 had $[\alpha]_D^{15} + 148°$ in water (*c*, 3·7); cf. $[\alpha]_D + 150°$, reported by Pazur & French (1952) for panose. M_1 had the same R_G in three solvents as an authentic sample of panose, and was not attacked by barley β-amylase or maltase-free saliva. The infrared spectrum of M_1 (panose), kindly determined by Dr D. M. W. Anderson, showed that it was present in the form 2 described recently by Wolfrom & Thompson (1957). M_1 (150 mg.) in water (3 ml.)

Table 1. *Properties of oligosaccharides synthesized from maltose by an extract of* Cladophora rupestris

The periodate-oxidation values are given in moles of formaldehyde/mole of oligosaccharide; theoretical values for the assigned structures are given in parentheses.

Oligosaccharide	Yield (g.)	R_G values *A*	R_G values *B*	DP	Periodate-oxidation value
M_0	0·03	0·38*	0·50*	1·94	—
M_1	2·16	0·18	0·28	—	1·0 (1)
M_2	2·11	0·35†	0·41†	3·04	2·7 (3)
M_3	0·01	0·07	0·14	2·63	—
M_4	0·31	0·11	0·17	3·72	1·8 (2)
M_5	0·47	0·19	0·26	3·95	4·1 (4)
M_6	0·02	0·03	0·09	3·69	—

* Authentic isomaltose had R_G values *A*, 0·38; *B*, 0·48.
† Authentic maltotriose had R_G values *A*, 0·33; *B*, 0·41.

Table 2. *Products of partial acid hydrolysis of the oligosaccharides and of the corresponding aldonic acids*

	Oligosaccharide hydrolysate*	Aldonic acid hydrolysate*	Tentative structure
M_1	M_0, maltose, glucose	M_0, glucose	Panose
M_2	Maltose, glucose	Maltose, glucose	Maltotriose
M_4	M_1, M_2, M_0, maltose, glucose	M_1, M_0, maltose, glucose	6^3-α-Glucosylmaltotriose†
M_5	M_2, maltose, glucose	M_2, maltose, glucose	Maltotetraose

* Unchanged oligosaccharide is not recorded.
† For full systematic name see text.

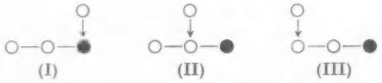
was reduced with potassium borohydride solution (4·5%, 1 ml.). After 2 hr. at room temperature, the solution was acidified with acetic acid (to decompose excess of borohydride), neutralized and evaporated to dryness. The product (120 mg.) was acetylated with sodium acetate (162 mg.) and acetic anhydride (3 ml.) at 135° for 20 min. The sugar acetate was isolated from ice–water and recrystallized from ethanol [m.p. and mixed m.p. with an authentic sample of panitol dodecaacetate, 148–149°; $[\alpha]_D^{15}+119°$ in chloroform (c, 1·0) (cf. +119° and +120° reported by Peat, Whelan & Edwards, 1955)]. M_1 is therefore identified as panose (6^2-α-glucosylmaltose).

Characterization of M_2 *as maltotriose*

M_2 had $[\alpha]_D^{15}+164°$ in water (c, 2·8); cf. $[\alpha]_D+160°$, reported by Whelan *et al.* (1953) for maltotriose. M_2 was slowly hydrolysed by maltase-free saliva and by barley β-amylase, giving glucose and maltose, as shown by paper-chromatography and reducing-power measurements. On incubation with D-enzyme, glucose and higher oligosaccharides were formed. The data reported in Tables 1 and 2 and the enzymic studies identify M_2 as maltotriose.

Characterization of M_4 *as* 6^3-α-*glucosylmaltotriose*

M_4 had $[\alpha]_D^{15}+177°$ in water (c, 0·5) and differed in R_G and M_G (electrophoretic mobility relative to that of D-glucose) values from maltotriose, maltotetraose, isomaltotriose and isomaltotetraose. It was not attacked by α- or β-amylase. On the assumption that M_4 is formed by trans-α-glucosylation from maltotriose, three possible structures have been considered:



(I) (II) (III)

where ○ and ● represent respectively non-reducing and reducing glucose residues, and the symbols — and ↓ represent 1 → 4- and 1 → 6-linkages.

On periodate oxidation at pH 8, the structures would yield 0, 1 and 2 moles of formaldehyde repectively. M_4 gave 1·8 moles. Structure (I) and the aldonic acid of compound (II) on partial acid hydrolysis could not yield panose. These results and those reported in Tables 1 and 2 therefore identify M_4 as 6^3-α-glucosylmaltotriose [*O*-α-D-glucopyranosyl-(1 → 6)-*O*-α-D-glucopyranosyl-(1 → 4)-*O*-α-D-glucopyranosyl-(1 → 4)-D-glucopyranose].

Characterization of M_5 *as maltotetraose*

M_5 had $[\alpha]_D^{15}+175°$ in water (c, 1·3); cf. $[\alpha]_D$ values of +176° and +177° reported by Whistler & Hickson (1955) and Whelan *et al.* (1953) for maltotetraose. M_5 was rapidly hydrolysed by maltase-free saliva to give maltose and glucose, and by barley β-amylase to give maltose. The enzymic studies and the data in Tables 1 and 2 characterize M_5 as maltotetraose.

Attempted transfer of α-glucosyl radicals to other sugars

Digests were prepared containing maltose (300 mg.), acceptor sugar (300 mg.), *C. rupestris* preparation (20 mg.) and 0·1 M-acetate buffer (pH 5·6, 2 ml.), and were examined at intervals, after incubation at 35°, by paper chromatography. The following acceptor sugars were tested: D-xylose, L-xylose, *N*-acetylglucosamine and L-sorbose. In all digests the same series of oligosaccharides were produced as were present in a control digest containing only maltose and extract.

DISCUSSION

The ability of many carbohydrases to catalyse both the hydrolysis and synthesis of oligosaccharides is now widely recognized (cf. Baumann & Pigman, 1957). The reactions may be formulated as follows:

$$\text{G–O–X} + \text{Enzyme-H} \rightarrow \text{Enzyme-G} + \text{H–O–X}, \quad (1)$$

$$\text{Enzyme-G} + \text{H–O–R} \rightarrow \text{Enzyme-H} + \text{G–O–R}, \quad (2)$$

where G–O–X represents a glucoside (the glucosyl donor) and H–O–R the acceptor molecule. When R is a hydrogen atom, hydrolysis takes place; if R is a univalent radical the synthesis of a new glucoside with the same configuration as the glucosyl donor occurs.

The present study shows that extracts of *C. rupestris* catalyse the synthesis of oligosaccharides from maltose when the concentration of water is sufficiently low. The main products of enzyme action, which have been characterized by chemical and enzymic methods, are panose (6^2-α-glucosylmaltose), maltotriose, 6^3-α-glucosylmaltotriose and maltotetraose. In addition, very small amounts of other oligosaccharides are also formed.

Although the yields of the oligosaccharides recorded in Table 1 are not quantitative, they are a

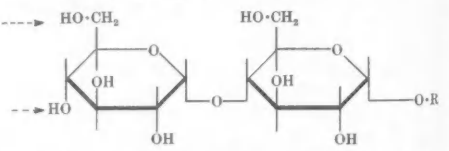


Fig. 1. Carbohydrate acceptors for α-glucosyl radicals transferred from maltose by extracts of *C. rupestris*. The arrows denote possible points of attachment of α-glucosyl radicals. R represents a hydrogen atom (maltose) or a glucose residue (maltotriose).

Table 3. *Trans-α-glucosylation: oligosaccharides synthesized from maltose by various enzyme preparations*

Biological source	Oligosaccharides	Reference
Penicillium chrysogenum Q. 176	Isomaltose, isomaltotriose, panose, 6^2-α-isomaltosylmaltose	Saroja, Venkataraman & Giri (1955)
Aspergillus niger (strain 152)	Isomaltose, isomaltotriose, panose	Barker & Carrington (1953)
Aspergillus oryzae	Isomaltose, isomaltotriose, panose, 6^2-α-isomaltosylmaltose	Pazur & French (1952)
Escherichia coli	Maltotriose and higher maltosaccharides	Barker & Bourne (1952)
Rat liver	Maltotriose, maltotetraose	Giri, Nagabhushanam, Nigam & Belavadi (1955)

measure of the relative proportion of the various sugars present in the equilibrium between synthesis and hydrolysis, under our particular experimental conditions (with 13% maltose solution). They show that the main transfer of α-glucosyl residues from maltose is to the hydroxyl group at $C_{(4)}$ or $C_{(6)}$ of the non-reducing end-group in maltose or maltotriose (Fig. 1). Glucose or panose do not appear to act readily as acceptor molecules.

These results may be compared with similar studies on trans-α-glucosylation by enzyme preparations from other biological sources, in which maltose was used as the α-glucosyl donor (Table 3).

It will be noted that the mould enzymes readily transfer α-glucosyl radicals to the primary alcoholic group in glucosaccharides, including glucose and panose, whereas the animal and bacterial enzyme systems can transfer only to the hydroxyl group at $C_{(4)}$ of the non-reducing end-group of the acceptor.

Experiments in this Laboratory (unpublished work) have also shown that the acceptor specificity for α-glucosyl radicals of the *C. rupestris* enzyme system differs from that found with cell-free extracts of the protozoan *Tetrahymena pyriformis* and with extracts of brewer's yeast.

In a further study of this acceptor specificity, the action of the algal extract on a mixture of maltose and a second sugar has been examined by paper chromatography. If the second sugar functions as an acceptor, new oligosaccharides will be formed in addition to those described here. As reported previously (Duncan *et al.* 1956), trans-α-glucosylation to D-galactose, D-fructose (and methanol) could not be detected. Similar experiments with D-xylose, L-xylose, L-sorbose and *N*-acetylglucosamine were unsuccessful. These six sugars do not therefore fulfil the acceptor-specificity requirements of the maltose trans-α-glucosylase. The results with the pentose sugars are in contrast with those obtained in an analogous study of trans-β-glucosylation in which cellobiose was used as β-glucosyl donor, since β-glucosylpentose disaccharides are formed in the presence of pentoses (D. J. Manners & J. L. Thompson, paper in preparation).

SUMMARY

1. An extract of *Cladophora rupestris* catalysed the synthesis of higher oligosaccharides from maltose.

2. The oligosaccharides were separated by charcoal–Celite chromatography and four sugars characterized by chemical and enzymic methods. They are panose, maltotriose, 6^3-α-glucosylmaltotriose [*O*-α-D-glucopyranosyl-(1 → 6)-*O*-α-D-glucopyranosyl-(1 → 4)-*O*-α-D-glucopyranosyl-(1 → 4)-D-glucopyranose] and maltotetraose.

3. The above and other evidence shows that, with the *C. rupestris* extract, maltose and maltotriose can function as α-glucosyl acceptors, whereas D-glucose, D-fructose, D-galactose, D-xylose, L-xylose, L-sorbose, *N*-acetylglucosamine and panose cannot.

4. The metabolism of maltose by transglucosylases from various biological sources is discussed.

We wish to thank Professor E. L. Hirst, F.R.S., for his advice and encouragement, and Dr W. J. Whelan for the gift of samples of maltotriose, panose and panitol acetate. The work described in this paper forms part of a research programme sponsored by the Institute of Seaweed Research, to whom W.A.M.D. is indebted for a research grant.

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The Effects of Manganese on the Solute Content of Rat-liver Mitochondria

By W. BARTLEY and J. E. AMOORE
Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, University of Oxford

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Manganese is known to affect the metabolic activities of isolated mitochondria (see e.g. Lindberg & Ernster, 1954) and also to prevent the loss of ultraviolet-light-absorbing substances from the mitochondria (Siekevitz & Potter, 1955). In view of these observations it was thought desirable to study the effect of Mn^{2+} ions on isolated mitochondria under a variety of conditions.

MATERIALS AND METHODS

Special chemicals

Sodium 1-azo-2-hydroxy-3-(2:4-dimethylcarboxanilido)-naphthalene-1′-(2-hydroxybenzene-5-sulphonate), used in the estimation of Mg^{2+} ions by the method of Mann & Yoe (1956), was purchased from LaMotte Chemical Products Co., Baltimore, Maryland, U.S.A. Ethylenediaminetetra-acetic acid (EDTA), formaldoxime hydrochloride and 8-hydroxyquinoline (AnalaR) were obtained from British Drug Houses Ltd.

Mitochondrial fractionation

This followed the procedure of Werkheiser & Bartley (1957). The mitochondrial pellet was suspended in sufficient 0·25 M-sucrose to give about 350 mg. wet wt. of mitochondria/ml. Usually 1 ml. of this suspension was added to 4 ml. of 0·25 M-sucrose or 4 ml. of 0·125 M-KCl, either of which contained $MnCl_2$, usually m-molar in the final suspension. After incubation, a portion (2 ml.) of the mixture was centrifuged, usually for 10 min. at 24 000 *g*. The supernatant fluid was decanted into 15 ml. conical centrifuge tubes containing 1 ml. of 30% trichloroacetic acid. After clarification by centrifuging the supernatant was set aside for analysis. The treatment of the mitochondrial pellet for the determination of the acid-soluble solutes and the acid-insoluble dry matter was described by Werkheiser & Bartley (1957). All analytical data refer to the samples (2 ml.) which were taken for centrifuging.

Analytical methods

Potassium was measured with a lithium internal standard flame photometer (Amoore, Parsons & Werkheiser, 1958). Manganese was measured by the use of formaldoxime (Bartley, Notton & Werkheiser, 1957). Calcium was estimated flame-photometrically with the apparatus of Exley (to be published) and chloride by the method of Sanderson (1952) with the slight modifications introduced by Amoore & Bartley (1958). The ultraviolet absorption at 260 mμ of the $HClO_4$ extracts of the supernatant medium was measured in a quartz cell with a 1 cm. light path in a Beckman spectrophotometer. From these measurements the absorption due to these substances in the original medium was calculated. The changes in absorption at this wavelength were assumed to be due to changes in the nucleotide content of the solutions. The estimation of the water content, and of the dry matter non-extractable with trichloroacetic acid, of the mitochondrial pellet was according to Werkheiser & Bartley (1957). Total phosphate was estimated by the method of Berenblum & Chain (1938), as modified by Bartley (1953), on a sample of the extract after wet-ashing according to Hanes & Isherwood (1949).

Measurements of pH were made with the Beckman probe assembly, type 14400, and a Beckman pH meter, model G. Measurements were made on the suspensions remaining after the sample (2 ml.) had been abstracted for centrifuging. The temperature of measurement was that at which the mitochondria had been incubated.

Magnesium was estimated either by the modification of the colorimetric method of Mann & Yoe (1956) described by Amoore & Bartley (1958) or by the flame-photometric method of Exley (to be published). Mn^{2+} ion interferes in

the colorimetric method by decolorizing the dye. The interference could be overcome either by causing the Mn^{2+} ions to form a complex with 8-hydroxyquinoline and extracting it into $CHCl_3$ (a slight modification of the procedure of Mann & Yoe, 1956) or by causing it selectively to form a complex with EDTA.

Removal of Mn^{2+} *ions by extraction.* To the extract in trichloroacetic acid (0·1 ml. of the mitochondrial extract or 0·5 ml. of the supernatant) in a 10 ml. graduated tube was added 0·5 ml. of conc. HNO_3. The mixture was slowly evaporated to dryness (taking about 2 hr.) and the residue was taken up in 2 ml. of 5 mM-HCl. To this was added 1 ml. of borate buffer (0·08 M-sodium borate, pH 9·2) and 0·1 ml. of 5% 8-hydroxyquinoline in $CHCl_3$. Chloroform (5 ml.) was immediately added and the tube was stoppered and shaken vigorously for about 5 sec. The lower $CHCl_3$ layer was carefully withdrawn by means of a Pasteur pipette and discarded. The extraction with $CHCl_3$ was repeated a further four times, then the magnesium dye was added and the procedure continued according to the modification of the method of Mann & Yoe (1956) without further addition of borate buffer.

Removal of Mn^{2+} *ions by ethylenediaminetetra-acetic acid.* To the samples of tissue extract was added sufficient EDTA to chelate all the manganese in the sample. It is immaterial if a slight excess of EDTA is added. Instead of the normal amount of calcium (0·2 μmole) 0·3 μmole was added to remove any excess of EDTA and still leave sufficient free Ca^{2+} ions to produce the enhancement of colour described by Mann & Yoe (1956; see also Amoore & Bartley, 1958). The sample was adjusted to 2 ml. and the subsequent procedure was that used for Mg^{2+} ions in the absence of Mn^{2+} ions.

Use of Titan yellow for the estimation of Mg^{2+} *ions in the presence of sucrose or* Mn^{2+} *ions.* In some earlier experiments a Titan yellow method (Garner, 1946; see also Siekevitz & Potter, 1955) for the estimation of Mg^{2+} ions was found to give erroneous results in the presence of sucrose or Mn^{2+} ions. Recoveries as low as about 61% occurred when the sucrose content of the test sample was 112 μmoles and only 90% recovery was obtained in the presence of 7·5 μmoles of sucrose. The phenomenon was not further studied because the more convenient and sensitive method of Mann & Yoe (1956) became available. Siekevitz & Potter (1955) do not mention any interference by sucrose; it is possible that there is no interference when polyvinyl alcohol is the stabilizer instead of gum ghatti. Whereas the interference by sucrose is comparatively small, the interference due to Mn^{2+} ions is gross and unpredictable. Mn^{2+} ion gives an apparent reaction for Mg^{2+} ion with Titan yellow. This is due to the formation of a dark precipitate, probably MnO_2, on the addition of the concentrated alkali. In a mixture of Mn^{2+} and Mg^{2+} ions the absorptions of the two ions in the Titan yellow test are not additive and an unpredictable optical density may be obtained. If the amount of Mn^{2+} ions added was large in comparison with the Mg^{2+} ions added the Mg^{2+} ions were grossly overestimated, but if the amounts of the two ions were more nearly equal, a large overestimation did not occur but, in some cases, an underestimation. It is not known if the use of Titan yellow, according to the procedure of Orange & Rhein (1951), as used by Siekevitz & Potter (1955), results in the same errors.

Duplicate estimations of K^+, Cl^-, Mn^{2+} and Mg^{2+} ions, phosphate, dry matter and water content agreed to better than 2% under the experimental conditions. According to Dr D. Exley (personal communication) duplicate estimations of Ca^{2+} and Mg^{2+} ions by his flame-photometric technique differ by not more than $\pm 3\%$ at the levels found in our experimental material.

RESULTS

Effect of manganese on the colour of the mitochondrial pellet. Mitochondria freshly prepared and sedimented at 0° yield a pale-buff pellet. On warming to room temperature the colour of the pellet changes within 1–2 min. to dark brown. As the brown colour develops, the mitochondrial pellet becomes increasingly translucent. On the other hand, when the mitochondria were obtained as a sediment from suspension media containing mM-Mn^{2+} ions, the pellet only very slowly turned brown on warming, taking up to 3 hr. when the suspension medium was 0·25 M-sucrose with Mn^{2+} ions and a somewhat shorter time when the medium contained 0·1 M-KCl and 0·05 M-sucrose. Thus the Mn^{2+} ions seem to preserve the mitochondria from disintegration.

Effect of manganese on the release of ultraviolet-light-absorbing material and on change of mitochondrial dry weight during incubation in 0·25 M-*sucrose solution.* The experiment shown in Table 1

Table 1. *Effect of manganese on the leakage of nucleotides from mitochondria*

Rat-liver mitochondria were prepared and suspended in 0·25 M-sucrose and were incubated at 21° alone or with the addition of $MnCl_2$ (final concn. mM). Samples of the suspension were centrifuged at the times given for subsequent analysis of the mitochondrial pellet and the medium. All analytical data refer to the samples (2 ml.) taken for centrifuging. The optical density was measured on the deproteinized solution and values were then expressed in terms of the volume of the original undiluted medium.

Incubation time (min.)	Concn. of Mn^{2+} ions in medium (mM)	Dry wt. of pellet (%)	Calc. optical density of medium at 260 mμ after precipitation of protein
0	0	33	0·277
5	0	32·3	0·510
10	0	27·9	0·895
15	0	27·6	1·224
20	0	26·9	1·287
40	0	26·6	1·305
60	0	26·6	1·326
0	1	36·6	0·194
5	1	38·0	0·364
10	1	38·0	0·382
15	1	34·3	0·370
20	1	33·1	0·390
40	1	30·4	0·478
60	1	31·7	1·104

confirms the results of Siekevitz & Potter (1955) that in the presence of mM-Mn^{2+} ions the leakage of substances absorbing at 260 mμ was greatly reduced. Table 1 also shows that the addition of the Mn^{2+} ions caused an immediate decrease in the water content of the mitochondrial pellet. Throughout the incubation, the % dry wt. of the mitochondrial pellet in the solution containing Mn^{2+} ions (cf. Tapley, 1956) was higher. In a similar experiment shown in Table 2 it was further shown that mM-Mn^{2+} ions also retarded the loss of material (presumably largely protein) precipitable with trichloroacetic acid from the mitochondria.

Effect of manganese on the release of ultraviolet-light-absorbing material and on the percentage of mitochondrial dry weight during incubation in solutions containing 0·05 M-sucrose and 0·1 M-potassium chloride. *In vivo*, the mitochondria are exposed to the high intracellular concentration of potassium, and in most metabolic experiments *in vitro* the mitochondria are suspended in media containing potassium. In these ionic media the

Table 2. *Manganese uptake of mitochondria and loss of nucleotide and protein*

The mitochondria were prepared and incubated as in Table 1. Optical density is expressed as in Table 1.

Incubation time (min.)	Mn^{2+} ions added to medium (μmoles)	Dry wt. of pellet (%)	Mn^{2+} ions in medium after incubation (μmole)	Calc. optical density of medium at 260 mμ after precipitation of protein	Protein in medium after incubation (mg.)
0	0	28·61	0	0·313	1·7
5	0	27·06	0	0·479	2·5
10	0	27·29	0	0·411	3·3
15	0	28·02	0	0·501	4·4
20	0	28·19	0	0·540	4·8
40	0	27·7	0	0·617	7·5
60	0	26·8	0	0·699	8·8
0	2	32·1	0·75	0·259	1·2
5	2	30·38	0·75	0·304	1·6
10	2	29·69	0·76	0·338	1·7
15	2	29·51	0·795	0·348	2·1
20	2	29·54	0·776	0·369	2·5
40	2	29·41	0·82	0·450	3·4
60	2	29·34	0·785	0·487	4·0

Table 3. *Effect of manganese on dry weight and nucleotide content of rat-liver mitochondria suspended in 0·1 M-potassium chloride and 0·05 M-sucrose*

The mitochondria were prepared in 0·25 M-sucrose, resuspended in the KCl–sucrose medium and incubated at the temperatures given below. Optical density is expressed as in Table 1. Analytical data refer to the samples (2 ml.) taken for centrifuging.

Temp.	Incubation time (min.)	Mn^{2+} ions added to medium (μmoles)	Dry wt. of pellet (%)	Calc. optical density of medium at 260 mμ
0°	0	0	29·46	0·738
	10	0	29·92	0·960
	20	0	29·8	1·044
	40	0	28·8	1·2
	60	0	28·61	1·416
20·7	5	0	28·18	1·374
	10	0	27·98	1·794
	20	0	26·82	2·658
	30	0	24·08	3·234
0	0	2	31·49	1·044
	10	2	31·21	1·128
	20	2	30·8	1·158
	40	2	30·47	1·284
	60	2	30·1	1·662
20·7	5	2	30·2	1·554
	10	2	30·16	2·256
	20	2	28·23	3·468
	30	2	23·55	3·918

Table 4. *Effect of manganese concentration on its uptake by rat-liver mitochondria suspended in 0·25 M-sucrose solution*

The mitochondria were prepared and maintained in 0·25 M-sucrose at 0°. The water content of the mitochondria before addition of Mn^{2+} ions was 2·83. Manganese chloride was added to give the amounts shown below and the mixture was kept at 0° for 15 min. before centrifuging. Analytical data refer to the sample (2 ml.) taken for centrifuging.

Mn^{2+} ions added to medium (μmoles)	Content of Mn^{2+} ion of mitochondrial pellet (μmoles)	Content of Mn^{2+} ion of dry mitochondrial pellet (m-moles/kg.)	Concn. of Mn^{2+} ion in water of pellet (m-molal)	Concn. of Mn^{2+} ion in medium (m-molal)	Concn. of Mn^{2+} ion in pellet/concn. of Mn^{2+} ions in medium	Water content of mitochondria (l./kg. dry wt.)
2	1·43	25·6	9·66	0·37	26·1	2·66
4	2·44	43·0	18·6	0·96	19·4	2·31
10	3·58	60·0	27·4	3·83	7·2	2·20

behaviour of the mitochondria is different from that in sucrose alone; for example, their adenosine triphosphatase activity stimulated by dinitrophenol is greatly enhanced (Lardy & Wellman, 1953). Accordingly, the effect of Mn^{2+} ions was tested in a medium containing 0·05 M-sucrose and 0·1 M-KCl. As shown in Table 3, Mn^{2+} ions no longer prevented the loss from the mitochondria of substances absorbing at 260 mμ (E_{260} substances). The rate of loss of these substances was greatly accelerated by raising the temperature of the suspending medium to 20°. At 0°, Mn^{2+} ions still maintained the water content of the mitochondrial pellet at a lower level than the control pellets without Mn^{2+} ions; this also occurred on incubation at 20°, but if incubation was prolonged beyond 20 min. at this temperature, both sets of mitochondria swelled to the same water content.

Uptake of manganese by mitochondria suspended in 0·25 M-sucrose solution. Siekevitz & Potter (1955) gave no measurements of the distributions of Mn^{2+} ions between the particles and the medium. The data of Maynard & Cotzias (1955) suggest that there would be little uptake of Mn^{2+} ions by the mitochondria *in vitro*. However, our experiments showed that more than one-half (1·25 μmoles, see Table 2, zero time with Mn^{2+} ions) of the Mn^{2+} ions was taken up by the mitochondria in the 5 min. at 0° that elapsed between adding the Mn^{2+} ions and warming the suspension to 20°. Incubation at this temperature did not result in a further uptake of Mn^{2+} ions.

Siekevitz & Potter (1955) showed that mitochondria suspended in media containing Mn^{2+} ions at a higher concentration than 2 mM lost their ability to carry out oxidative phosphorylation. In Table 4 is shown the uptake of Mn^{2+} ions by mitochondria from solutions containing 1, 2 and 5 mM concentrations of this ion. The amount of Mn^{2+} ions taken up increased with the external concentration but not proportionally to it. The maximum concentration gradient was obtained with the lowest concentration of Mn^{2+} ions used. These results contrast with those of Maynard & Cotzias (1955), who found only a 10% uptake of Mn^{2+} ions at the very low concentration of Mn^{2+} ions (0·03 mM) that they used. However, it will be shown that prolonged incubation at room temperature in the absence of substrate leads to a loss of the Mn^{2+} ions that was previously taken up (see Fig. 1), and this may explain their low uptake.

As shown already, the addition of Mn^{2+} ions to the mitochondria resulted in a loss in the water content of the pellet. More water was lost when the content of Mn^{2+} ions was increased, but there was no simple relationship between loss and the external concentration of Mn^{2+} ions (Table 4).

Uptake of manganese by mitochondria suspended in 0·1 M-potassium chloride and 0·05 M-sucrose. As Mn^{2+} ions had no effect on the loss of E_{260} substances when the mitochondria were suspended in the KCl-containing medium, it might have been expected that uptake of Mn^{2+} ions from this medium was inhibited. However, as shown in Table 5, the uptake of Mn^{2+} ions from this medium was much greater than from sucrose alone, reaching 35–40 m-moles of Mn^{2+} ions/kg. of dry mitochondrial pellet in between 10 and 20 min., compared with 25 m-moles of Mn^{2+} ions/kg. of dry mitochondrial pellet in mitochondria incubated for the same time in 0·25 M-sucrose. After 1 hr. at 0° the content of Mn^{2+} ions of the pellet had risen to 57 m-moles/kg. dry wt.

At 21° the rate and amount of uptake of Mn^{2+} ions were still further accelerated. Within 5 min., more than 60% of the added Mn^{2+} ions were taken up and after 30 min. the content of Mn^{2+} ions reached 81 m-moles/kg. dry wt. of mitochondria. If this amount of Mn^{2+} ions is considered to be dissolved in the mitochondrial water the concentration is 23·3 mM, a ratio (Mn^{2+} ions of mitochondria: Mn^{2+} ions of medium) of 185:1. In the experiments so far described the mitochondria were added to a mixture of KCl and $MnCl_2$ solutions and thus the two cations might have competed for penetration of the mitochondria. The uptake of Mn^{2+} ions by mitochondria that had been equilibrated with 0·1 M-KCl before the addition of Mn^{2+} ions was

Table 5. *Uptake of manganese by mitochondria suspended in* 0·1 M-*potassium chloride and* 0·05 M-*sucrose*

The mitochondria were prepared and incubated as in Table 3.

Incubation time (min.)	Temp.	Concn. of Mn^{2+} ions in water of		Content of Mn^{2+} ions of dry mitochondrial pellet (m-moles/kg.)	Concn. of Mn^{2+} ions in pellet/concn. of Mn^{2+} ions in medium
		Pellet (m-molal)	Supernatant (m-molal)		
0	0°	10·9	0·63	24·8	17·3
10	0	15·0	0·51	34·6	29·5
20	0	17·5	0·42	41·2	41·4
40	0	22·2	0·29	53·1	76·0
60	0	23·4	0·24	56·9	95·7
5	21	18·3	0·37	44·0	49·6
10	21	23·4	0·26	57·1	88·5
20	21	25·3	0·17	67·8	150
30	21	23·3	0·13	81·0	185

Table 6. *Effect of prior equilibration with* 0·1 M-*potassium chloride and* 0·05 M-*sucrose on the uptake of manganese by mitochondria*

The mitochondria were prepared in 0·25 M-sucrose and then added to 0·125 M-KCl solution to give a final concentration in the suspending medium of 0·1 M-KCl and 0·05 M-sucrose. After equilibration for 15 min., $MnCl_2$ was added to separate portions of the suspension to give the concentrations shown below.

Time (min.)	Temp.	Mn^{2+} ions added to medium (μmoles)	Content of Mn^{2+} ions of dry mitochondrial pellet (m-moles/kg.)	Concn. of Mn^{2+} ions in water of		Concn. of Mn^{2+} ions in pellet/concn. of Mn^{2+} ions in medium	Water content of mitochondria (l./kg. dry wt.)
				Pellet (m-molal)	Supernatant (m-molal)		
15	0°	1	24·8	5·8	0·23	25·2	4·28
15 + 11	0 | 24	1	25·8	5·1	0·3	17·0	5·05
15	0	2	33·1	8·4	0·6	14·0	3·93
15 + 11	0 | 24	2	39·8	9·4	0·53	17·7	4·25
15	0	3	43·3	10·4	0·91	11·4	4·17
15 + 11	0 | 24	3	57·5	13·4	0·81	16·6	4·27
15	0	4·2	51·4	12·5	1·49	8·4	4·11
15 + 11	0 | 24	4·2	66·8	17·3	1·3	13·3	3·86
15	0	8·7	66·2	16·5	3·4	4·9	4·00
15 + 11	0 | 24	8·7	93·4	23	3·3	7·0	4·05

also tested (Table 6). The amount of Mn^{2+} ions taken up was very similar to that taken up when the KCl and Mn^{2+} ions were added simultaneously. This shows that potassium and manganese did not compete. However, the mitochondria swelled during the preliminary treatment with KCl, and not all this water was expelled on the addition of Mn^{2+} ions. Thus the concentration gradients were considerably less than when the two salts were added together. This finding suggests that the mitochondrial mass rather than the concentration gradient determines the amount of Mn^{2+} ions taken up. More Mn^{2+} ions were taken up at higher temperatures.

Effect of anaerobiosis on uptake of manganese. The extra uptake of Mn^{2+} ions that occurred in mitochondria incubated at 21° could have been due either to metabolism or to an 'adsorption' process with a high activation energy (chemisorption). Any such chemisorption process is unlikely to be affected by the presence of oxygen. In Fig. 1 is shown the time course of uptake of Mn^{2+} ions by mitochondria aerobically and anaerobically at 25° and aerobically at 0°. The mitochondria at 0° were added to the chilled medium contained in a measuring cylinder which was stoppered, the contents were mixed and it was placed in a bath of ice–water. The mitochondria which were to be

Table 7. *Effect of ethylenediaminetetra-acetic acid on the uptake of manganese by mitochondria*

The mitochondria were prepared and maintained in 0·25 M-sucrose at 0°. Manganese chloride (1·8 μmoles) and the amounts of EDTA given below were added to a series of samples (2 ml.) of the suspension. After 15 min. the samples were centrifuged to separate the mitochondrial pellet for analysis. The water content of the mitochondrial pellet before the addition of Mn^{2+} ions was 2·49 l./kg. of dry mitochondrial pellet.

EDTA added (μmoles)	Content of Mn^{2+} ions of pellet (μmoles)	Concn. of Mn^{2+} ions in water of		Concn. of Mn^{2+} ions in pellet/concn. of Mn^{2+} ions in medium	Water content of pellet (l./kg. dry wt.)
		Pellet (m-molal)	Supernatant (m-molal)		
0	1·15	13	0·37	35·2	2·23
0·4	1·03	11·4	0·42	26·9	2·28
1·0	0·73	8·0	0·55	14·5	2·27
1·94	0·3	3·1	0·72	4·3	2·42
4·0	0·022	0·24	0·93	0·26	2·34
9·6	0·023	0·23	0·86	0·27	2·56

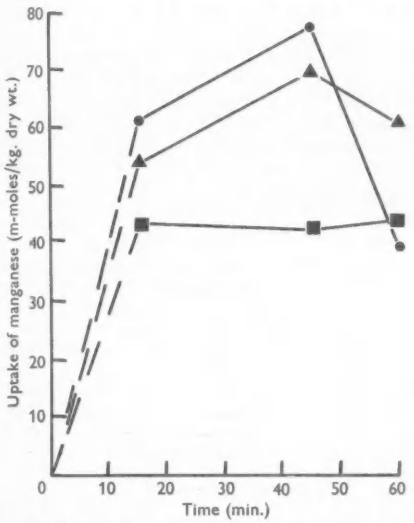

Fig. 1. Effect of anaerobiosis on uptake of manganese by mitochondria. The preparation and incubation of the mitochondria are described in detail in the text. ●, Incubation at 25° in O_2; ▲, incubation at 0° in air; ■, incubation at 25° in N_2.

incubated at 25° were added to Warburg vessels which were filled with O_2 or N_2 at 0°. The mitochondria were tipped from the side arm immediately before putting the manometers in the water bath at 25°. The manometers were removed from the bath at the appropriate times (15, 45 and 60 min.) and a sample (2 ml.) of the vessel contents was centrifuged immediately at high speed to separate the mitochondria from the medium. As shown in Fig. 1, there was a large uptake of Mn^{2+} ions during the first 15 min. under all three conditions, but the amount in N_2 was only about 69% of that in O_2 at the same temperature. Further incubation did not cause an increase in the content of Mn^{2+} ions of the anaerobic mitochondria. In the presence of O_2 there was an increase during the next 30 min. (61–76 m-moles of Mn^{2+} ions/kg. dry wt.) followed by a loss. The mitochondria maintained at 0° showed changes in content of Mn^{2+} ions which were similar to, but less in amount than, those shown by the mitochondria incubated at 25° in O_2.

These observations suggested that whereas part of the uptake of Mn^{2+} ions by mitochondria may be accounted for by an adsorption process there may be an additional uptake by active processes.

Effect of ethylenediaminetetra-acetic acid on uptake of manganese by mitochondria. EDTA is a strong chelator of Mn^{2+} ions and is frequently added to mitochondrial preparations, as it is reported to stabilize enzyme systems (see Slater & Cleland, 1953; Beaufay, Hers, Berthet & de Duve, 1954). When the action of EDTA on uptake of Mn^{2+} ions was studied, it was found that it strongly competed with the mitochondria for the added Mn^{2+} ions (Table 7). A concentration of 0·002 M-EDTA completely inhibited uptake of Mn^{2+} ions. When allowance is made for the interstitial fluid occluded in the mitochondrial pellet (about one-third of the total fluid of the pellet), it can be calculated from the data in Table 7 that about 2·6 μmoles of EDTA were necessary to chelate the 1·8 μmoles of Mn^{2+} ions added to the medium.

Changes of pH *during uptake of manganese by mitochondria.* The pH of mitochondrial suspensions stored in 0·25 M-sucrose or in sucrose–KCl mixtures remains constant at about 7·2. The uptake of Mn^{2+} ions by mitochondria is accompanied by a fall in the pH of the suspension. Table 8 shows that the suspension became more acid with increasing amounts of Mn^{2+} ions added to the suspension. The most probable explanation is that Mn^{2+} ions are bound to a site on the mitochondrial structure by a chelating mechanism which results in the displacement of a H^+ ion. An even larger fall in pH

occurred when mitochondria suspended in sucrose were added to a KCl–$MnCl_2$ mixture (Table 8, Expt. 2). This is probably correlated with the larger uptake of Mn^{2+} ions that occurred in this medium (compare Tables 4 and 5). The change in pH apparently varied with the metabolic state of the mitochondria. Thus the fall in pH was slightly less when the suspension was warmed up in N_2, markedly less when the suspension was warmed in O_2 and was only transitory when substrate was added (Table 8). The reason for this is obscure, as the binding of Mn^{2+} ions is greater when the temperature is raised. Table 9 shows the balance of ion movements that occurred when $MnCl_2$ at different concentrations was added to mitochondria in 0·25 M-sucrose solution. The production of acid was estimated indirectly by titrating a separate sample of mitochondrial suspension with HCl and noting the amount of acid needed to lower the pH to the levels previously observed on addition of $MnCl_2$.

As shown in Table 9, there was an overall loss of cation from the mitochondria. This was probably compensated for by a loss of phosphates (see Amoore & Bartley, 1958). It may be concluded that the uptake of Mn^{2+} ions is mainly compensated for by the release of H^+ ions and that the final balance is achieved by a small uptake of Cl^- ion and the loss of K^+ and Mg^{2+} ions from the mitochondria. It is possible that the movements of the last two ions are secondary effects of the change in pH, for the addition of HCl alone to produce a similar change in pH caused a large loss of K^+ ions and some of Mg^{2+} ions, as shown in the last line of Table 9.

Changes in content of potassium chloride of mitochondria on incubation with manganese chloride. When mitochondria in 0·25 M-sucrose solution are added to a KCl–$MnCl_2$ mixture (to give finally 0·1 M-KCl, 0·05 M-sucrose and 0·001 M-$MnCl_2$), Cl^- ion is found at a considerably lower concentration (Table 10) in the mitochondria than in the medium, but when the $MnCl_2$ is added after pre-incubation for 15 min. with KCl, both the Cl^- and K^+ ions freely enter the mitochondria. If the Mn^{2+} ions are bound to the same sites under the two different conditions, it is not at all clear why the movements of Cl^- ions should be so different. Under both conditions prolongation of incubation time at 0° or elevation of the temperature (Table 11) resulted in a net entry of chloride.

Table 8. *Changes in* pH *during uptake of* Mn^{2+} *ions by mitochondria*

In Expt. 1 the mitochondria were prepared and incubated as in Table 1. In Expt. 2, at 25°, the mitochondria were shaken in Warburg vessels and a portion (2 ml.) of the suspension was removed for centrifuging at the times given below.

Expt. no.	Incubation medium	Other conditions	Temp.	Concn. of Mn^{2+} ions (m-molal)	Incubation time (min.)	pH of suspension
1	0·25 M-Sucrose	—	0°	0	—	7·2
	0·25 M-Sucrose	—	0	1	15	6·8
	0·25 M-Sucrose	—	0	2	15	6·6
	0·25 M-Sucrose	—	0	5	15	6·4
2	0·25 M-Sucrose	—	0	0	—	7·2
	0·1 M-KCl + 0·05 M-sucrose	—	0	1	15	6·1
					45	6·0
					60	6·2
	0·1 M-KCl + 0·05 M-sucrose	Gassed with N	25	1	15	6·2
					45	6·3
					60	6·4
	0·1 M-KCl + 0·05 M-sucrose	Gassed with O_2	25	1	15	6·7
					60	6·9
	0·1 M-KCl + 0·05 M-sucrose	Gassed with O_2 and 200 μmoles of sodium succinate added as substrate	25	1	15	6·9
					45	7·4
					60	7·5

Table 9. *Summary of the ion movements compensating for uptake of manganese*

The mitochondria were prepared and separated as described in Table 1. They were incubated for 15 min. before centrifuging. $\Sigma = (\Delta Mn^{2+} + \Delta K^+ + \Delta Mg^{2+}) - (\Delta Cl^- + \Delta H^+)$, where Δ = change in mitochondrial content of the ion or change of titratable acid in the suspension.

Additions to medium	Change in ion content (Δ) of mitochondrial pellet (m-equiv./kg. dry wt.)				Acid produced (m-equiv./kg. dry wt.)	Σ all ions (m-equiv.)
	Mn^{2+}	Cl^-	K^+	Mg^{2+}	H^+	
mM-$MnCl_2$	+50	+3	−15	−12	44	−24
2 mM-$MnCl_2$	+86	+7	−15	−8	69	−13
5 mM-$MnCl_2$	+120	+24	−15	−22	79	−20
mM-HCl		−1	−25	−6	(36)	−30

Table 10. *Effect of manganese on potassium and chloride content of mitochondria at 0°*

In Expts. 1, 2 and 4, the mitochondria were prepared in 0·25 M-sucrose and added to a mixture of KCl and $MnCl_2$ to give the concentrations stated below. In Expt. 3, the Mn^{2+} ions were added after the mitochondria had equilibrated with 0·1 M-KCl and 0·05 M-sucrose for 15 min. The excess of each ion is the amount remaining, after deduction from the total, of the amount that would be in solution in the mitochondrial water if the ion were at the same concentration as in the external medium.

Expt. no.	Time (min.)	Medium	Concn. of Mn^{2+} ions (m-molal)	Excess of K^+ ions (m-moles/kg. of dry mitochondrial pellet)	Excess of Cl^- ions (m-moles/kg. of dry mitochondrial pellet)
1	11·5	0·1 M-KCl + 0·05 M-sucrose	1	0	−68
	60		1	0	−49
2	10	0·1 M-KCl + 0·05 M-sucrose	1	−10	−60
3	0	0·1 M-KCl + 0·05 M-sucrose	0	−5	−54
	15		0	16	−5
	15		1	13	53
	50		1	42	71
4	15	0·25 M-Sucrose	1	35	7·8

Table 11. *Effect of manganese on potassium and chloride content of mitochondria on incubation*

In Expt. 1 the mitochondria were prepared in 0·25 M-sucrose and added to a mixture of KCl and $MnCl_2$, as in Table 10 (Expts. 1 and 2), and in Expt. 2 the Mn^{2+} ions were added after equilibration with 0·1 M-KCl and 0·05 M-sucrose for 15 min. Concn. of Mn^{2+} ions was mM.

Expt. no.	Time (min.)	Temp.	Excess of K^+ ions (m-moles/kg. of dry mitochondrial pellet)	Excess of Cl^- ions (m-moles/kg. of dry mitochondrial pellet)
1	11·5	20°	4	−57
	60	20	28	−45
2	11	23·8	52	95
	22·5	23·8	46	62

Table 12. *Effect of manganese on the magnesium content of mitochondria*

The mitochondria, prepared in 0·25 M-sucrose, contained 30·3 m-moles of Mg^{2+} ions/kg. At zero time the mitochondria, suspended in 0·25 M-sucrose, were added to KCl soln. to give a final concn. in the medium of 0·1 M-KCl and 0·05 M-sucrose. After 20 min., 9 ml. of the suspension was added to tubes containing varying amounts of $MnCl_2$. At 35 min. half of the contents of each tube was poured into beakers of 15 ml. capacity and shaken in a Dubnoff Metabolic Shaking Incubator (Precision Scientific Co., Chicago, Ill., U.S.A.) at 24°. Samples were centrifuged for analysis at the times given below.

Concn. of Mn^{2+} ions in medium (mM)		0	0·5	1	1·5	2·0	5·0	0	0·5	1·0	1·5	2·0	5·0
		Content of Mn^{2+} ions of mitochondria (m-moles/kg. dry wt.)						Content of Mg^{2+} ions of mitochondria (m-moles/kg. dry wt.)					
Time (min.)	Temp.												
15	0°	0	—	—	—	—	—	14·6	—	—	—	—	—
35	0	0	24·8	33·1	43·1	51·4	66·2	8·2	9·9	10·9	12·5	15·3	9·8
46	24	0	25·8	39·8	57·5	66·8	93·4	9·3	6·4	7·0	10·3	6·5	6·1
57·5	24	0	16·6	36·1	52·5	87·0	82·6	9·4	6·3	7·5	14·4	7·4	6·0
70	0	0	21·9	32·5	41·2	53·3	77·1	9·1	9·7	9·1	10·0	9·6	10·0

Effect of manganese on the content of Mg^{2+} *and* Ca^{2+} *ions of the mitochondria.* Several experiments (when the unreliable Titan yellow method was still used) indicated that the usual content (30–35 m-moles/kg.) of Mg^{2+} ions of mitochondria prepared in 0·25 M-sucrose was reduced to somewhat less than half (see also Siekevitz & Potter, 1955) on adding the particles to a KCl–$MnCl_2$ mixture (final concentrations: 0·1 M-KCl, 0·05 M-sucrose, 0·001 M-$MnCl_2$). Subsequently, these experiments were repeated by adding the mitochondria first to KCl solution and then, after about 15 min., to the $MnCl_2$ solution. The estimations were done with the modification of the method of Mann & Yoe (1956) described under Methods, with the use of 8-hydroxyquinoline to extract the Mn^{2+} ions. Table 12 shows that in this mitochondrial preparation most of the endogenous Mg^{2+} ions were lost

Table 13. *Effect of ethylenediaminetetra-acetic acid on the uptake of manganese by mitochondria and on their content of* Mg^{2+} *and* Ca^{2+} *ions*

The mitochondria were prepared and incubated as in Table 1, except that the amounts of EDTA given below were added to the incubation medium. Incubation time, 5 min. at 0°.

Concn. of EDTA in medium (m-molal)	Concn. of Mn^{2+} ions in medium (m-molal)	Content of Mn^{2+} ions of dry mitochondrial pellet (m-moles/kg.)	Content of Mg^{2+} ions of dry mitochondrial pellet (m-moles/kg.)	Content of Ca^{2+} ions of dry mitochondrial pellet (m-moles/kg.)
0	0	0	11·2	31·5
0	1	29	6·8	13·7
0·2	1	26·1	8·6	14·5
0·5	1	18·4	7·6	16·0
0·97	1	7·5	11·5	14·0
2·0	1	0·6	7·8	6·7
4·8	1	0·6	8·2	5·4

during the first 15 min. after the addition of the KCl solution at 0°; it fell to a minimum by 35 min. and thereafter there was a slight uptake of the Mg^{2+} ions, which were retained for a further 50 min. The addition of Mn^{2+} ions, 15 min. after the KCl, inhibited the loss of Mg^{2+} ions. All concentrations of Mn^{2+} ions were not equally effective in preventing the loss of Mg^{2+} ions, the most effective being about 1·5 mM. On the other hand, at 24° Mn^{2+} ions caused an increase in the loss of Mg^{2+} ions except at the optimum concentration of Mn^{2+} ions (1·5 mM), where there was an uptake of Mg^{2+} ions with time. By contrast, as shown by Table 11, Mn^{2+} ions causes a loss of Mg^{2+} ions from mitochondria in sucrose, and this was about equal to the extra quantity of Mg^{2+} ions that was displaced by Mn^{2+} ions at 24° subsequent to treatment with KCl. Even when the level of endogenous Mg^{2+} ions of the mitochondria was low (Table 13), Mn^{2+} ions still caused the same displacement of Mg^{2+} ions from mitochondria suspended in 0·25 M-sucrose.

The calcium content of our mitochondrial preparations is fairly constant at between 25 and 30 m-moles/kg. of dry mitochondria. This calcium was not displaced when the mitochondria were suspended in a mixture of 0·1 M-KCl, 0·05 M-sucrose and mM-$MnCl_2$. It was, however, displaced when mM-$MnCl_2$ was added to a mitochondrial suspension in 0·25 M-sucrose (Table 13, compare lines 1 and 2). It is of interest that 0·2 mM-EDTA displaced an almost identical amount of calcium from the same preparation of mitochondria as did the mM-$MnCl_2$. The addition of both 0·2 mM-EDTA and mM-$MnCl_2$ did not result in an additive effect. Further calcium was displaced when sufficient EDTA had been added to chelate all the added manganese (Table 13, lines 6 and 7). It appears probable from these observations that the calcium, which is easily displaced from the mitochondria, is located on the outer mitochondrial surface.

Effect of manganese on the leakage of phosphate compounds from the mitochondria at 0°. An earlier section of this paper has shown that nucleotide-like substances leak from the mitochondria on storage at 0° and that this may be prevented in some circumstances by the addition of Mn^{2+} ions. Also, Amoore & Bartley (1958) have shown a progressive leakage of phosphate esters from rat-liver mitochondria stored at 0° in either 0·25 M-sucrose or in 0·125 M-KCl. Table 14 shows that leakage of phosphate from the mitochondria was practically abolished by the addition of 1·5 mM-$MnCl_2$ to the medium.

Table 14. *Effect of manganese on the leakage of phosphate from mitochondria at* 0°

The mitochondria were prepared in 0·25 M-sucrose and then added to 0·125 M-KCl to give a final concentration in the suspending medium of 0·1 M-KCl and 0·05 M-sucrose. After equilibration for 15 min. at 0°, $MnCl_2$ was added to separate portions of the suspension to give the concentrations stated below. The mitochondria initially contained 83·1 m-moles of phosphate/kg. dry wt.

Time (min.)	Incubation medium	Excess of phosphate content (m-moles/kg. of dry mitochondria)
0	0·1 M-KCl + 0·05 M-sucrose	73·6
20		67·6
35		50·0
70		49·0
15	0·1 M-KCl + 0·05 M-sucrose + 0·5 mM-$MnCl_2$	61·1
50		57·8
15	0·1 M-KCl + 0·05 M-sucrose + mM-$MnCl_2$	61·6
50		58·3
15	0·1 M-KCl + 0·05 M-sucrose + 1·5 mM-$MnCl_2$	66·8
50		65·8
15	0·1 M-KCl + 0·05 M-sucrose + 5 mM-$MnCl_2$	58·4
50		57·3

Effect of manganese on the leakage of phosphate compounds from the mitochondria at 24°. As shown in Table 15, raising the temperature to 24° resulted

in a greater loss of phosphate from mitochondria than occurred at 0°. This is consistent with the observation often made that mitochondria become metabolically inactive more rapidly at elevated temperatures than at 0°. Table 15 also shows that even 0·5 mM-$MnCl_2$ slowed the rate of phosphate loss. When the $MnCl_2$ concentration added was mM, not only was the loss of phosphate inhibited, but there was an uptake of the endogenous phosphate that had previously leaked from the mitochondria. With 1·5 mM-$MnCl_2$ the content of phosphate of the mitochondria was restored to the original concentration found when the particles were first isolated in sucrose solution. As at 0°, 5 mM-$MnCl_2$ was not so effective as 1·5 mM-$MnCl_2$ in preventing loss of phosphate or in causing uptake of phosphate. Continued incubation of the mitochondria in the presence of Mn^{2+} ions at 24° resulted in a loss of phosphate. This loss was minimal when the $MnCl_2$ was added at 1·5 mM concentration. It appears probable that both the extra uptake of Mn^{2+} ions and of phosphate that occurred on raising the temperature from 0° to 24° were the result of active processes.

Table 15. *Effect of manganese on the leakage of phosphate from mitochondria at 24°*

The mitochondria were those of Table 14. At time 20 min. in Table 14 the mitochondria were warmed to 24° for the times stated below. The mitochondria initially contained 83·1 m-moles of phosphate/kg. dry wt.

Time (min.)	Incubation medium	Excess of phosphate content (m-moles/kg. of dry mitochondria)
11	0·1 M-KCl +	31·6
22·5	0·05 M-sucrose	38·5
11	0·1 M-KCl +	46·4
22·5	0·05 M-sucrose + 0·5 mM-$MnCl_2$	39·2
11	0·1 M-KCl +	80·6
22·5	0·05 M-sucrose + 1 mM-$MnCl_2$	74·1
11	0·1 M-KCl +	86·1
22·5	0·05 M-sucrose + 1·5 mM-$MnCl_2$	84·2
11	0·1 M-KCl +	80·4
22·5	0·05 M-sucrose + 5 mM-$MnCl_2$	75·3

Manganese and mitochondrial swelling. Mitochondria sometimes swell in isotonic solutions and often swell in hypotonic solutions, the effect depending on the composition of the medium. The reason for the swelling in isotonic solutions is not known and in our experience the phenomenon is unpredictable. It is, however, more likely to occur when the temperature of the suspending medium is raised above 0°. The Mn^{2+} ion is able to prevent or slow down the rate of swelling of mitochondria over a wide range of conditions (see Fonnesu & Davies, 1956; Tapley, 1956). In our experiments manganese always caused a shrinkage in the mitochondria at 0° in 0·25 M-sucrose solution (Tables 1–4 and 16). Increasing concentration of

Table 16. *Effect of manganese on the water content of mitochondria at 0°*

Expt. no.	Time (min.)	Suspending medium	Initial content of Mn^{2+} ions of medium (mM)	Water content of mitochondria: Without Mn^{2+} ions (l./kg. dry wt.)	Water content of mitochondria: With Mn^{2+} ions (l./kg. dry wt.)
1	—	0·25 M-Sucrose	0	1·93	—
	0	0·1 M-KCl + 0·05 M-sucrose	0	2·39	—
	0		1	—	2·18
	60		0	2·50	—
	60		1	—	2·32
2	—	0·25 M-Sucrose	0	2·25	—
	2	0·1 M-KCl + 0·05 M-sucrose	1	—	2·10
3	—	0·25 M-Sucrose	0	2·27	—
	5	0·1 M-KCl + 0·05 M-sucrose	1	—	2·96
4	0	0·25 M-Sucrose	0	2·49	—
	0	0·1 M-KCl + 0·05 M-sucrose	1	—	2·23
5	—	0·25 M-Sucrose	0	2·48	—
	0	0·1 M-KCl + 0·05 M-sucrose	0	3·89	—
	15		0	4·2	—
	50		0	4·36	—
	15		0·5	—	4·28
	50		0·5	—	4·77
	15		1·0	—	3·93
	50		1·0	—	3·95
	15		1·5	—	4·17
	50		1·5	—	4·31
	15		5·0	—	4·00
	50		5·0	—	4·16

Mn^{2+} ions in the medium (Table 4) increased the loss of mitochondrial water, and no water was lost when sufficient EDTA was added to the medium to chelate all the Mn^{2+} ions (Table 7). On raising the temperature to 20–25° the mitochondria swelled, whether Mn^{2+} ions were present or not, but the swelling was less when Mn^{2+} ions were present (Tables 1, 2 and 17). In saline media the findings are not so consistent. Usually when mitochondria in sucrose solution are transferred to a saline medium of equivalent osmotic pressure at 0°, there is a slight swelling (Table 16). If the saline medium also contains Mn^{2+} ions, the swelling is usually less and on occasions the mitochondria shrink to a water content less than that of the mitochondria in sucrose solution (Table 16, Expt. 2); compare also Fonnesu & Davies (1956). When the Mn^{2+} ions were added after equilibration with 0·1 M-KCl, swelling was still prevented, but there was no simple relationship between the amount of Mn^{2+} ions added and the inhibition of swelling.

DISCUSSION

The findings reported in this paper clearly show that metabolic behaviour and permeability of mitochondria in solutions which are mainly non-ionic are different from those of mitochondria in largely ionic media. In general, mitochondria appear to be somewhat more permeable in ionic solutions and more liable to loss of endogenous solutes, for example, magnesium and phosphate compounds. The addition of manganese to the suspending medium in optimum amounts appears to delay or reverse this 'ageing' process. The manganese is more effective in this role in the non-ionic media as judged by the rate at which E_{280} substances leak from the mitochondria. The data are consistent with the idea that saline solutions produce an increase in the permeability of the mitochondrial membrane to anions. Manganese in some way tends to prevent this increase in permeability. This is best illustrated by considering the results on the distribution of chloride. In sucrose solutions the uptake of manganese is not matched by an equivalent uptake of chloride and a shrinkage of the mitochondria occurs. A similar phenomenon occurs when magnesium chloride or hydrochloric acid is used instead of manganese chloride. All these observations are consistent with a relatively slow permeation of the mitochondrial membrane by the Cl^- ion. After treatment with potassium chloride solution under the conditions described in Table 10 and the subsequent addition of manganese chloride, the uptake of manganese and chloride is more or less equivalent and no shrinkage occurs. The swelling of mitochondria exposed to the action of the salt solutions alone is reduced by the addition of Mn^{2+} ions. In one case (Table 16, Expt. 2) there was actually a shrinkage in the mitochondria compared with those suspended in 0·25 M-sucrose. Once again chloride was found at a lower concentration in the pellet than in the medium. This argument implies that the shrinkage of mitochondria in the presence of manganese is a secondary effect due to the impermeability of the mitochondrial membrane to the external chloride.

It is considered unlikely that the manganese taken up by the mitochondria is in free solution and is osmotically active, because the intramitochondrial fluid would then be grossly hypertonic and the mitochondria would swell. It is much more likely that the manganese is bound to the mitochondria by some form of adsorption. It is suggested that the amount of manganese taken up reflects the amount of mitochondrial protein accessible to the manganese. Thus the initial rapid uptake that occurs at 0° is probably adsorption upon the mitochondrial membrane, whereas the slower uptake with time represents a slow penetration of the manganese to sites within the mitochondria. The greater uptake of manganese occurring in mainly ionic media represents the greater permeability of the mitochondrial membrane that occurs in these media. It is clear from the work of Stanbury & Mudge (1953) on the exchange of potassium in mitochondria, and from the observations on loss of nucleotide given in this paper, that the permeability of the mitochondrial membrane is increased during metabolism. Thus it might be expected that the uptake of manganese would also be increased by increase of temperature, and hence of metabolism, by the increased accessibility to binding sites

Table 17. *Effect of manganese on the water content of mitochondria incubated at 24°*

The incubation medium was 0·1 M-KCl + 0·05 M-sucrose. The incubation conditions were as described in Table 3.

Expt. no.	Time (min.)	Initial content of Mn^{2+} ions of medium (mM)	Water content of mitochondria: Without Mn^{2+} ions (l./kg. dry wt.)	Water content of mitochondria: With Mn^{2+} ions (l./kg. dry wt.)
1	15	0	2·25	—
	15	1	—	2·31
	30	0	3·15	—
2	11	0	5·6	—
	22·5	0	6·3	—
	11	0·5	—	5·05
	22·5	0·5	—	5·39
	11	1·0	—	4·25
	22·5	1·0	—	4·43
	11	1·5	—	4·27
	22·5	1·5	—	4·24
	11	5·0	—	4·05
	22·5	5·0	—	4·17

within the mitochondria. Two observations argue against this simple explanation of uptake of manganese. The first is the difference occurring aerobically and anaerobically, and the second the loss of manganese that occurred during the later stages of incubation at 25°. It is feasible that the mitochondria may not be so permeable under anaerobic conditions as they are aerobically, and this may, in turn, reflect the difference in metabolism under the two conditions. The loss of manganese occurring during prolonged incubation may merely be a measure of protein leakage from the mitochondria. Any other explanation would seem to involve a more direct coupling of metabolism with uptake of manganese ('active transport') and this cannot at present be excluded.

It seems unlikely that the effects of manganese recorded in this paper can apply to the mitochondria *in vivo*, as the total liver content of manganese, even if all were concentrated in the mitochondria, amounts to only about 1–5 % of the amount taken up by the mitochondria in mM-manganese chloride, although even at such low levels the amount of Mn^{2+} ions present may condition the permeability of the mitochondrial membrane.

Whatever the means whereby manganese functions in the stabilization of the mitochondrial membrane, it appears that its uptake is dependent on the presence of free Mn^{2+} ions, for the uptake was completely abolished by the addition of an equivalent amount of ethylenediaminetetra-acetic acid.

Although most of the observations on movements of ions resulting from the addition of manganese chloride to a mitochondrial suspension can be explained as the consequence of a change in permeability of the mitochondrial membrane, it is not easy to explain the recovery of endogenous phosphate and magnesium which occurred on incubation of the mitochondria with 1·5 mM-manganese chloride. It is difficult to escape the conclusion here that Mn^{2+} ions are closely associated with the active uptake process.

The large uptake of manganese by mitochondria that has been demonstrated emphasizes the danger in assuming that in cell-free preparations the added solutes are evenly distributed throughout the suspension. The possibility of such localization of solutes needs to be borne in mind when deducing the probable direction of reaction sequences from thermodynamic data and overall concentrations of reactants.

SUMMARY

1. At 20°, mM-manganese chloride decreased the loss of ultraviolet-light-absorbing nucleotide-like substances and protein from mitochondria suspended in 0·25 M-sucrose. Manganese did not affect nucleotide loss when the suspending medium was 0·1 M-potassium chloride plus 0·05 M-sucrose. In either medium, mM-manganese chloride maintained the mitochondria at a higher dry weight than in its absence.

2. Mitochondria suspended in 0·25 M-sucrose and mM-manganese chloride took up manganese at a high rate at 0°, reaching about 25 m-moles/kg. dry weight of mitochondria. The uptake was increased when the concentration of manganese chloride in the medium was increased, but not proportionally. With 5 mM-manganese chloride the uptake reached 60 m-moles/kg. dry weight. The loss of water from the mitochondria was higher in the presence of 5 mM-manganese chloride than with mM.

3. The uptake of manganese by mitochondria suspended in 0·1 M-potassium chloride, 0·05 M-sucrose and mM-manganese chloride at 0° was about 1·5 times as fast as in 0·25 M-sucrose. A further increase of 1·5 times in the rate of uptake occurred when the temperature was raised to 20°. The total uptake reached 81 m-moles/kg. dry weight. The uptake of manganese was about the same when the manganese chloride was added after the mitochondria had equilibrated with the potassium chloride–sucrose mixture.

4. After incubation for 15 min. in nitrogen at 25°, the uptake of manganese of mitochondria suspended in 0·1 M-potassium chloride plus 0·05 M-sucrose was only 69 % of the uptake in oxygen. Further incubation caused an increase of the manganese content of the mitochondria in oxygen but not of those in nitrogen.

5. The uptake of manganese by mitochondria suspended in 0·25 M-sucrose and mM-manganese chloride was completely inhibited by 1·5 mM-ethylenediaminetetra-acetic acid.

6. The addition of manganese chloride to a mitochondrial suspension caused a fall in the pH of the suspension of as much as 1 pH unit. The change of pH was increased by an increase of manganese concentration and by the presence of potassium chloride in the medium and decreased by raising the temperature above 0°. In 0·25 M-sucrose solution, between 60 and 90 % of the uptake of manganese could be accounted for by the liberation of H^+ ions.

7. Manganese maintained the impermeability of the mitochondrial membrane to chloride.

8. At 0°, mM-manganese chloride displaced about one-third of the endogenous magnesium content and about one-half of the endogenous calcium content. Transfer of mitochondria from 0·25 M-sucrose to 0·1 M-potassium chloride plus 0·05 M-sucrose caused a loss of about half the endogenous magnesium, but no loss of the endogenous calcium. Manganese prevented the loss of

magnesium at 0° which occurs in the salt–sucrose mixture. The most effective concentration was 1·5 mM. Incubation of mitochondria at 24° in 0·1 M-potassium chloride, 0·05 M-sucrose and 1·5 mM-manganese chloride after they had lost endogenous magnesium at 0°, resulted in an uptake of most of the magnesium lost.

9. The loss of mitochondrial phosphate that occurred in 0·1 M-potassium chloride plus 0·05 M-sucrose at 0° was abolished by the addition of 1·5 mM-manganese chloride to the medium. Incubation at 24°, in the manganese-containing medium, of mitochondria which had already lost phosphate resulted in a restoration of the phosphate content.

10. In sucrose or saline media at 0° or at 25° the presence of mM-manganese chloride resulted in a lower water content of mitochondria than occurred in its absence.

11. The mode of action of manganese in the stabilization of mitochondria is discussed.

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The Esterase Activity of the Fibrinolytic System

By M. LASSEN
Biological Institute of the Carlsberg Foundation, and University Institute of Physical Chemistry, Copenhagen, Denmark

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The solubilization of fibrin clots in mammalian blood is caused by a proteolytic enzyme termed plasmin. This enzyme is not present in significant concentrations in normal plasma, but it can be produced from its precursor, plasminogen, through the interaction of appropriate activating substances.

The most potent and commonly used activator of human plasminogen preparations is streptokinase. This substance is found in the culture filtrate from certain strains of β-haemolytic streptococci (Tillett & Garner, 1933). Investigations by Kaplan (1944) and Christensen (1945) revealed the activating properties of the streptococcal product. The conclusion that the conversion of plasminogen into plasmin is catalysed by streptokinase was reached by Christensen & McLeod (1945), and although the reaction has later proved to be more complicated (Geiger, 1952; Müllertz & Lassen, 1953; Sherry, 1954; Asahina & Oka, 1954), it is still probable that the last step in the reaction sequence, the conversion of plasminogen into plasmin, is enzymic.

Müllertz & Lassen (1953) suggested that streptokinase is unable to activate pure human plasminogen, but that an activator of plasminogen is formed through a reaction between streptokinase and a substance, termed proactivator, which is present in large amounts in human plasma. This concept was accepted by Troll & Sherry (1955), but was later rejected by Sherry, who now presumes identity between plasminogen and proactivator and supposes the activator to be a complex of streptokinase with plasminogen or plasmin (Sherry & Alkjaersig, 1956).

It seems to be commonly accepted that the formation of activator proceeds through a stoicheiometric reversible reaction between streptokinase

and 'proactivator'. This was clearly shown in qualitative experiments by Troll & Sherry (1955), and in entirely different quantitative experiments by Müllertz (1955).

Experiments performed by Müllertz (1954) and M. Lassen (unpublished work) indicated that the mechanism of the streptokinase activation was even more complicated than was suggested by the above-mentioned investigators. However, the necessary condition of an experimental approach to these complicated problems is the availability of more accurate methods for determining the components in question. Troll, Sherry & Wachman (1954) found that streptokinase-activated human globulin resembles trypsin in being able to catalyse the hydrolysis of L-lysine esters and esters of L-arginine with a bound α-amino group. Later experiments performed by Troll & Sherry (1955) relate most of the lysine esterase activity to the activator, and the arginine esterase activity plus the remaining lysine esterase activity to plasmin. The following scheme summarizes the suggestions proposed:

$$\text{Streptokinase} + \text{proactivator} \rightleftarrows \underset{\text{(lysine esterase activity)}}{\text{activator}}$$

$$\text{Plasminogen} \xrightarrow{\text{activator}} \text{plasmin (arginine esterase activity).}$$

Troll & Sherry found arginine esters well suited for the estimation of plasmin since, as compared with the protein substrates generally used, inhibitors had little effect on their hydrolysis. Accordingly, benzoyl-L-arginine ethyl ester was used as a plasmin substrate in the present investigations. The hydrolysis of this and of L-lysine ethyl ester was followed in the Warburg apparatus by measuring the carbon dioxide produced from bicarbonate. This procedure is highly accurate and allows estimations of the amounts of substrate split without interrupting the reaction.

MATERIALS AND METHODS

Benzoyl-L-arginine ethyl ester. (BAEe.) The hydrochloride of this compound was prepared according to the method of Bergman, Fruton & Pollok (1939) as modified by Schwert & Eisenberg (1949). The melting point was 130° (uncorrected), as found by Schwert & Eisenberg. This ester showed no spontaneous hydrolysis under the experimental conditions used here.

L-Lysine ethyl ester. (LEe.) The hydrochloride of this compound was prepared according to the method of Werbin & Palm (1951). The ester showed little or no spontaneous hydrolysis under the test conditions.

Streptokinase. Varidase was kindly placed at my disposal by the Lederle Laboratories Division, American Cyanamid Co. The units are those stated on the packages according to Christensen (1949).

Human plasminogen and proactivator. Plasma (1 vol.) was mixed with 4 vol. of acetone at 0°, and the precipitate washed with acetone and ether. The dried powder obtained was easily soluble and no deterioration of active compounds (except of plasmin inhibitors: T. Astrup, paper in preparation) had occurred. The yield was 60–80 mg./ml. of plasma.

Bovine plasmin. Bovine plasminogen was activated with a mixture of streptokinase and human proactivator. Owing to the lability of purified bovine plasmin at neutral pH (Müllertz, 1955), a solution containing 1% of bovine fibrinogen, prepared according to Astrup & Müllertz (1952), was used as a source of plasminogen. Proactivator was supplied as human globulin prepared according to Milstone (1941) and redissolved to the original serum volume in diethylbarbiturate buffer, pH 7·8, ionic strength 0·15. The activation was performed at room temperature by adding 5 ml. of globulin solution and 10 000 units of streptokinase to 50 ml. of fibrinogen solution. A qualitative impression of the reaction's course was obtained by following the increasing fibrinolytic effect of the mixture. Samples of the mixture (0·1 ml.) were incubated for 3 min. at 37° with 2 ml. of diethylbarbiturate buffer, pH 7·8, containing 10 National Institute of Health units of thrombin. Then 1 ml. of 0·3% bovine fibrinogen was added, and lysis time was measured as the time which elapsed between this addition and the solubilization of the clot formed. After incubation of the streptokinase–globulin–fibrinogen mixture for 90–120 min. constant lysis times were obtained. The reaction was therefore stopped at 120 min. by the addition of 240 ml. of acetone, and the precipitate was dried with acetone and ether.

This preparation was easily soluble, and the plasmin was stable for 3 hr. at 25° and pH 7·34. The inevitable contamination by human plasmin constituted less than 5% of the total plasmin activity.

Manometric technique. The technique is that generally used (Parks & Plaut, 1953; Lundquist, Thorsteinsson & Buus, 1955). Acid liberated by hydrolysis of the substrate releases CO_2 from a CO_2–$NaHCO_3$ buffer. The CO_2 produced is measured manometrically in the Warburg apparatus. The assay was performed at 25° in 15 ml. Warburg vessels, with a liquid volume of 0·2 ml. in the side arm and 2 ml. in the flask.

Unless otherwise stated the following concentrations of esters and of the buffer constituents were used for determining the esterase activities. BAEe esterase (plasmin: the amounts are given in the legends to the figures): 25 mg. of BAEe giving a concentration of 0·033 M; 0·25 M-$NaHCO_3$ in the liquid phase and 50% (v/v) of CO_2 in the gas phase giving pH 7·34. Under these conditions the decrease in pH during the reaction was calculated as 0·03 when the whole manometer scale was utilized (equivalent to about 380 μl. of CO_2). LEe esterase (activator and plasmin; the amounts are given in the legends to the figures): 20 mg. of LEe giving a concentration of 0·037 M; 0·033 M-$NaHCO_3$ and 0·13 M-NaCl were present in the liquid phase and 100% of CO_2 in the gas phase. This gives pH 6·16 and an ionic strength of 0·27. The decrease in pH during the reaction was calculated at 0·12 when the whole manometer scale was utilized.

Calculation for the carbon dioxide–bicarbonate buffer

Solubility of carbon dioxide. This must be known for the calculation of the flask constants. The value 0·756 l./l. atm. for the solubility in pure water at 25° (*International Critical*

Tables, 1928) was used. However, since the solubility of CO_2 varies with the concentration of bicarbonate, this value is not correct, so the error in the flask constants caused by the use of the incorrect value was calculated. It amounted to about one-fifth of the error in the solubility coefficient for CO_2. The relative error was insignificant, and the value was therefore applicable.

Calculation of pH. The apparent first ionization constant (K_1) of carbonic acid is given by the expression

$$K_1 = \frac{(H^+)\,(HCO_3^-)}{(CO_2)},$$

in which the parentheses represent activities of the constituents enclosed.

At concentrations of bicarbonate higher than infinite dilution, the solubility of CO_2 is altered. However, the activity of CO_2 is proportional to the partial pressure of CO_2, and is therefore equal to the concentration in pure water at the same pressure.

The activity coefficient of HCO_3^- ion decreases when the concentration is increased, and K_1', as defined by

$$K_1 = \frac{(H^+)\,[HCO_3^-]}{(CO_2)},$$

will therefore increase with increasing concentration. (The parentheses represent activities and the brackets concentration of the constituents enclosed.) The corresponding decrease in pK_1' was calculated from determinations of pH in solutions containing different concentrations of $NaHCO_3$ and saturated with CO_2 at 25°. The partial pressure of CO_2 was equal to the barometric pressure corrected for the vapour pressure of water at 25°. The pH was measured potentiometrically with a glass electrode (type b from Radiometer, Copenhagen, which has no significant Na^+ ion error below pH 11–12) and a calomel reference electrode (Fig. 1). The experimental error was about ±0·01, which is a lower accuracy than usual in such investigations, but is satisfactory for practical work with the Warburg apparatus.

Influence of carbonate. All CO_2–$NaHCO_3$ buffers contain a certain amount of carbonate due to the reaction

$$HCO_3^- \rightleftarrows H^+ + CO_3^{2-}.$$

Most of the acid produced by hydrolysis of the substrate is consumed by the reaction

$$H^+ + HCO_3^- = H_2CO_3 = CO_2 + H_2O.$$

But a certain amount of the acid escapes estimation, since it is consumed by carbonate:

$$H^+ + CO_3^{2-} \rightarrow HCO_3^-.$$

In order to calculate the concentration of carbonate in a certain buffer, it is necessary to know the second ionization constant K_2' of carbonic acid, defined by

$$K_2' = \frac{(H^+)[CO_3^{2-}]}{[HCO_3^-]}.$$

(The parentheses represent activity and the brackets the concentrations of the constituents enclosed.)

The relation between pK_2' and the ionic strength (Fig. 2) was calculated from determinations of the pH in different concentrations of a Na_2CO_3–$NaHCO_3$ buffer at 25°. The pH was measured as above.

The concentration of carbonate in the initial state can now be calculated from the equation

$$\frac{K_2'}{K_1'} = \frac{(H^+)[CO_3^{2-}]}{[HCO_3^-]} \Big/ \frac{(H^+)[HCO_3^-]}{(CO_2)} = \frac{[CO_3^{2-}](CO_2)}{[HCO_3^-]^2}.$$

In the final state the increase in (CO_2) can be calculated from the increase in the pressure measured in the Warburg apparatus. Exact calculations of the decrease in the concentration of bicarbonate are laborious, but tests have shown that below pH 8 the amount of bicarbonate arising from the decrease in carbonate may be neglected and the change in bicarbonate concentration may be calculated from the output of CO_2 alone.

Values corrected as described were used in the experiments on the relation between pH and BAEe esterase activity. The corrections were unnecessary in the other experiments, where comparisons have been made only between results obtained at the same pH.

RESULTS AND DISCUSSION

Benzoyl-L-arginine ethyl ester esterase (plasmin)

With the concentration of BAEe used (0·033 M) the amount of substrate split is proportional to time and to the concentration of bovine plasmin

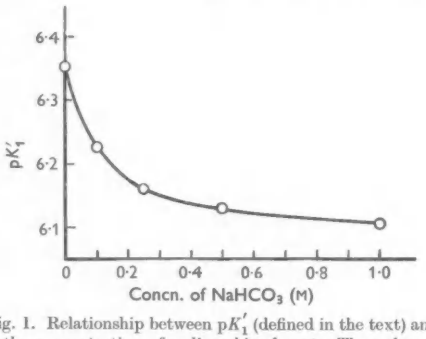

Fig. 1. Relationship between pK_1' (defined in the text) and the concentration of sodium bicarbonate. The value at infinite dilution is that found by Harned & Davis (1943).

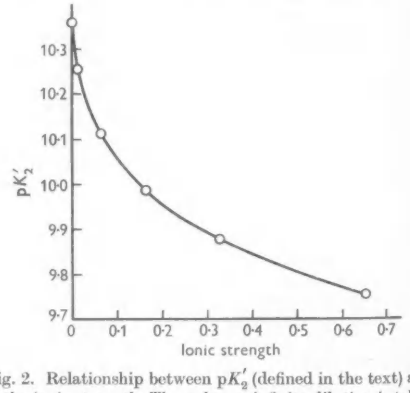

Fig. 2. Relationship between pK_2' (defined in the text) and the ionic strength. The value at infinite dilution is taken from *Handbook of Chemistry and Physics* (1948).

(Fig. 3). Similar results were obtained with human plasmin and with trypsin.

The relationship between reaction rate and substrate concentration is shown in Fig. 4, the maximum rate being reached at about 0·015 M-substrate concentration when 0·5 mg. of bovine plasmin was the enzyme source.

The reaction rate does not alter much between pH 7 and 8 (Fig. 5), but optimum activity seems to be at about pH 7·9 for both human and bovine plasmin. Troll *et al.* (1954) found the optimum at pH 9 for human plasmin with the same substrate. The difference may be due to the 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer used in their experiments.

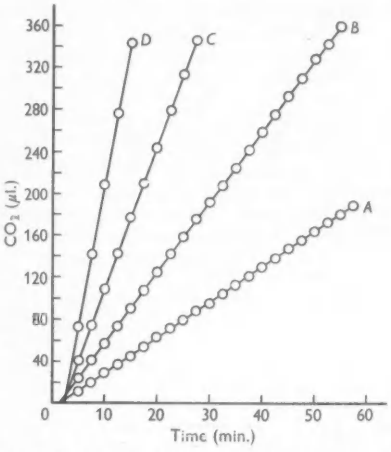

Fig. 3. Hydrolysis of BAEe with bovine plasmin. Temperature 25°; 50% CO_2 in the gas phase, giving pH 7·34, was used. In the main compartment of the flasks: 2·5 (*A*), 5 (*B*), 10 (*C*) or 20 (*D*) mg. of bovine plasmin dissolved in 2 ml. of 0·25 M-$NaHCO_3$. In the side arms: 25 mg. of BAEe dissolved in 0·2 ml. of 0·25 M-$NaHCO_3$.

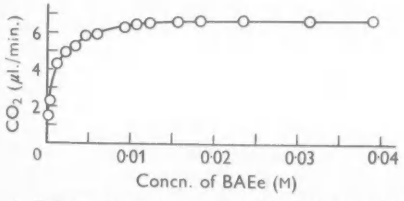

Fig. 4. Relationship between rate of hydrolysis and concentration of BAEe. Temperature 25°; 25% CO_2 in the gas phase, giving pH 7·64, was used. In the main compartment of the flasks: 30, 24, 18, 12 or 6 mg. of BAEe dissolved in 2 ml. of 0·25 M-$NaHCO_3$. In the side arms: 5 mg. of bovine plasmin dissolved in 0·2 ml. of 0·25 M-$NaHCO_3$.

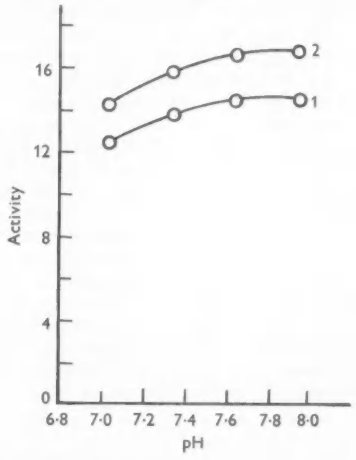

Fig. 5. Effect of pH on the rate of hydrolysis of BAEe. Temperature 25°. 0·25 M-$NaHCO_3$ in the liquid phase and 100, 50, 25 or 12·5% CO_2 in the gas phase, giving the pH values 7·04, 7·34, 7·64 or 7·94 respectively was used. Curve 1: bovine plasmin; ordinate = µl. CO_2/min. In the main compartment of the flasks: 10 mg. of bovine plasmin dissolved in 2 ml. In the side arms: 25 mg. of BAEe dissolved in 0·2 ml. Curve 2: human plasmin; ordinate = 10 × µl. of CO_2/min. In the main compartment of the flasks: 25 mg. of BAEe dissolved in 2 ml. In the side arms: 5 mg. of plasma acetone-dried powder + 1000 units of streptokinase dissolved in 0·2 ml. The observed values were corrected for the amounts of acid consumed by the conversion of carbonate into bicarbonate.

Determination of plasminogen in human plasma

Such determinations are complicated by the high content of plasmin inhibitors in plasma as well as in the preparation of streptokinase used for the activation. A simple estimation of plasmin activity in the activated sample will give no information of the amount of plasmin formed, since the amount of plasmin bound by inhibitors is quite unknown. The method described below is based upon the following two facts:

(1) The inhibitors present in the streptokinase preparation are ineffective in the present method. This is evident from the identical plasmin activities obtained when plasminogen in plasma is activated by a large excess of streptokinase and by twice this amount of streptokinase (Fig. 6, broken line).

(2) A preparation of human plasmin in which no activator and probably no inhibitors are present can easily be made by heating human globulin activated by streptokinase to 100° at pH 2 (Müllertz, 1955).

A determination of plasminogen in plasma can now be performed as follows: plasminogen is converted into plasmin by adding an excess of streptokinase, and the resulting plasmin activity measured (Fig. 6, broken line). Increasing amounts of the above-mentioned preparation of human plasmin are added to plasma, and the resulting activities measured (Fig. 6, curve *B*). The mixture of plasma and plasmin which has the same activity as plasma activated by streptokinase also contains the same amount of plasmin. Therefore a final determination of plasmin present in the heated preparation (Fig. 6, curve *A*) permits a calculation of the concentration of plasminogen in plasma in terms of μl. of CO_2/min./ml. of plasma (here 44·5). Further, it seems probable that a calculation of initial plasmin concentration and of inhibitor should be possible through more detailed analysis of such curves.

Lysine esterases (activator and plasminogen)

With the concentration of LEe used, the amount of substrate split is proportional to time and to the concentration of lysine esterase (solutions of streptokinase and plasma acetone-dried powder; Fig. 7).

Optimum activity was found at pH 6·1 (Fig. 8). With the selected buffer the pH decreases during the reaction from 6·16 to 6·04 when the whole manometer scale was utilized (corresponding to about 380 μl. of CO_2). The figure shows that only slight alterations in esterase activity take place during this shift of pH.

The straight lines obtained in Fig. 7 show the lysine esterase to be stable at pH 6·1 in the presence of LEe, but in the absence of LEe, lysine esterase was found to be labile, which is in accordance with the investigations on the stability of activator performed by Müllertz (1955). Since the manometric technique involves equilibration for 30 min. with the gas phase, the method can hardly be used for the estimation of preformed lysine esterase. To determine the amount of lysine esterase formed from given amounts of the precursor preparations the reaction must be started by adding one precursor, e.g. streptokinase, to a mixture of LEe and the other precursor. The slope of the linear part of the curve then gives the lysine esterase activity. This method was used to determine the amount of lysine esterase formed from constant amounts of acetone-dried powder of plasma and varying

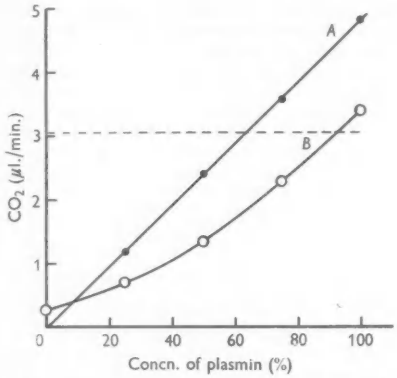

Fig. 6. Estimation of plasminogen in human plasma. Temperature 25°; 50 % CO_2 in the gas phase, giving pH 7·34, was used. In the side arms: 25 mg. of BAEe dissolved in 0·2 ml. of 0·25 M-$NaHCO_3$. In the main compartment of the flasks: broken line, 100 μl. of human plasma + 1000 units of streptokinase + 1900 μl. of 0·25 M-$NaHCO_3$. Esterase activity: 3·05 μl. of CO_2/min. With 2000 units of streptokinase instead of 1000 units the esterase activity was 3·08 μl. of CO_2/min. Curve *A*: 2 ml. of plasmin solution of the concentration denoted on the abscissa. The solution of plasmin was prepared as described by Müllertz (1955) except that the last precipitate was dissolved in 0·25 M-$NaHCO_3$. Before use 1 vol. of this solution was diluted with 2 vol. of 0·25 M-$NaHCO_3$. Curve *B*: 2 ml. of 0·25 M-$NaHCO_3$ containing the same concentration of plasma as that used for the broken line and the same concentrations of plasmin as those used for curve *A*.

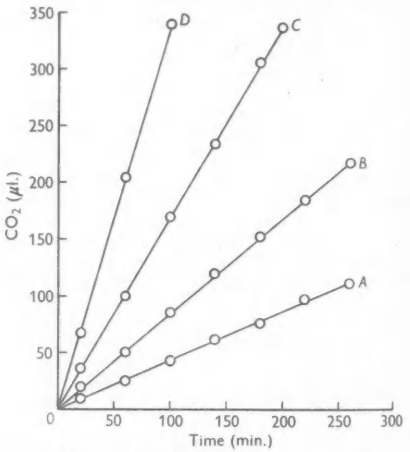

Fig. 7. Hydrolysis of LEe with solutions of streptokinase and acetone-dried powder of plasma. Temperature 25°; 100 % CO_2 in the gas phase. In the side arms: 20 mg. of LEe dissolved in the solution described. In the main compartment of the flasks: 0·25 (*A*), 0·5 (*B*), 1·0 (*C*) and 2·0 (*D*) ml. of a solution of 100 mg. of plasma acetone-dried powder + 10 000 units of streptokinase in 10 ml. of the solution described, plus the same solution to a total volume of 2 ml. The readings were started 5 min. after mixing.

amounts of streptokinase (Fig. 9). Assuming that the point of intersection between the ordinate and the extrapolated curve represents the plasmin activity of all samples, the relationship between the concentration of activator and streptokinase should be obtained by a displacement of the zero point of the co-ordinate system to this intersection point. The curve obtained like this resembles very closely the curve obtained by Müllertz (1955) showing the relation between the concentration of activator and of streptokinase in solutions containing a constant concentration of proactivator. Still keeping the above assumption the results are furthermore in excellent agreement with the interpretation that both plasmin and activator can hydrolyse LEe (Troll & Sherry, 1955), that the formation of activator proceeds as a stoicheiometric reaction (Troll & Sherry, 1955, Müllertz, 1955) and the formation of plasmin is a catalysed reaction (Christensen & McLeod, 1945). However, the assumption does not agree with the suggestion of Sherry & Alkjaersig (1956) that proactivator and plasminogen are identical, which means that plasminogen is the precursor to both activator and plasmin. Under these conditions the formation of increasing concentrations of activator with increasing concentrations of streptokinase should involve a simultaneous decrease in the concentration of plasmin, as indicated by the broken curve in Fig. 9. Assuming that this is correct, the results are still in accordance with the results obtained by the above-mentioned investigators. On the other hand, this assumption can hardly be reconciled with the fact that increasing concentrations of

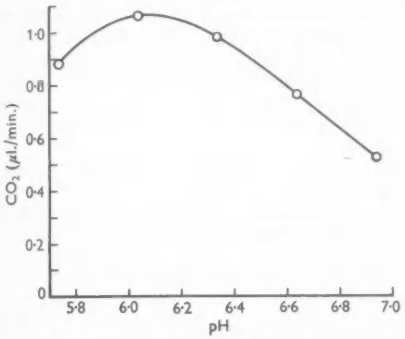

Fig. 8. Relationship between rate of hydrolysis of LEe and pH. Temperature 25°. For pH values above 6: liquid phase as described and 100, 50, 25 or 12·5% CO_2 in the gas phase, giving pH values 6·04, 6·34, 6·64 or 6·44 respectively was used. For pH 5·74: as for pH 6·04 except that NaCl was substituted for half of the $NaHCO_3$. In the main compartment of the flasks: 20 mg. of LEe + 5 mg. of plasma acetone-dried powder dissolved in 2 ml. In the side arms: 500 units of streptokinase dissolved in 0·2 ml. The points represent the slopes of the linear part of the curves.

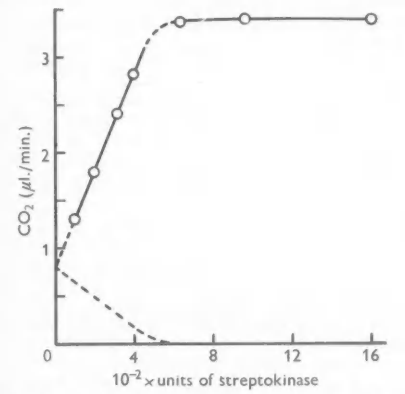

Fig. 9. Relationship between the concentration of lysine esterase and of streptokinase. Temperature 25°; 100% CO_2 in the gas phase. In the main compartment of the flasks: 20 mg. of LEe + 16 mg. of plasma acetone-dried powder dissolved in 2 ml. of the solution described. In the side arms: The amounts of streptokinase denoted on the abscissa dissolved in 0·2 ml. of the solution described. Points represent the slopes of the linear part of the curves. (The curve with 100 units of streptokinase is shown in Fig. 10.) Broken curve: for explanation, see text.

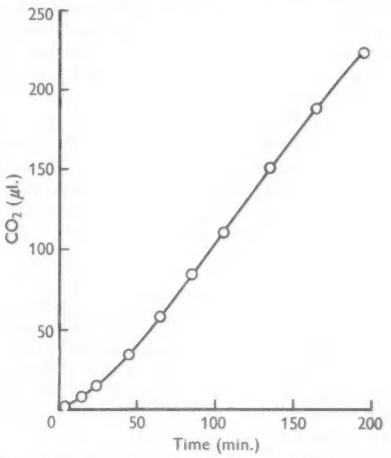

Fig. 10. Formation of lysine esterase in the presence of LEe. Temperature 25°; 100% CO_2 in the gas phase. In the main compartment of the flask: 20 mg. of LEe + 16 mg. of plasma acetone-dried powder dissolved in 2 ml. of the solution described. In the side arm: 100 units of streptokinase dissolved in 0·2 ml. of the solution described.

streptokinase do not reduce the BAEe esterase activity of human plasmin (legend to Fig. 6).

Fig. 10 shows the curve used to measure the amount of lysine esterase obtained with the smallest concentration of streptokinase in Fig. 9. It is obvious that the formation of lysine esterase was finished about 60 min. after the start. This interval of time was shorter in the mixtures used to obtain the remaining points of the curve in Fig. 9. Since the formation of activator is known to proceed very rapidly (Müllertz, 1955), and since LEe is known to inhibit the formation of plasmin but not the formation of activator (Troll & Sherry, 1955), it is most probable that the initial positive acceleration of the curve is due mainly to the slow transformation of plasminogen into plasmin. Apparently the lysine esterase is not quite stable in this mixture. This lack of stability was also found in all mixtures containing an excess of proactivator, whereas lysine esterase made with excess of streptokinase was found to be stable in the presence of LEe (Fig. 7).

Activation of plasminogen in the presence of benzoyl-L-arginine ethyl ester

The manometric technique has been used to follow the conversion of plasminogen into plasmin. Activator, bovine plasminogen and BAEe were mixed in the Warburg flask at zero time, and the hydrolysis of substrate was followed with time. [Experimental conditions were as follows. In the side arms: 2–8 mg. of plasma acetone-dried powder + 250–2000 units of streptokinase dissolved in 0·2 ml. of 0·25 M-$NaHCO_3$. In the main compartment of the flasks: 25 mg. of BAEe + 4–10 mg. of bovine plasminogen (prepared according to Müllertz, 1953) dissolved in 2 ml. of 0·25 M-$NaHCO_3$. A temperature of 25° and 100 % carbon dioxide in the gas phase, giving pH 7·04, were used.] The hydrolysis caused by the plasmin present in the activator preparation was estimated in separate experiments and subtracted from the values obtained. When these corrected results were plotted against time squared, straight lines were obtained. However, it was found that BAEe inhibited the activation rather strongly (contrary to the findings of Troll & Sherry, 1955). Since the concentration of BAEe decreased during the experiments the activator activity increased, and therefore it was impossible to interpret the experimental results.

SUMMARY

1. The esterase activity of the fibrinolytic system was measured with benzoyl-L-arginine ethyl ester or L-lysine ethyl ester as substrate. The acid formed was estimated by measuring the carbon dioxide liberated from bicarbonate solutions.

2. The relation of the first and second ionization constants of carbonic acid to the ionic strength was determined so as to make possible calculations on the carbon dioxide–bicarbonate buffer in the assay method.

3. A method of determining human plasminogen in the presence of plasmin inhibitors is described.

4. The relationship between the concentration of lysine esterase and of streptokinase is established, and the results are discussed in connexion with those obtained by other investigators.

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The Biochemistry of Organotin Compounds

TRIALKYLTINS AND OXIDATIVE PHOSPHORYLATION

By W. N. ALDRIDGE
Unit for Research in Toxicology, M.R.C. Laboratories, Carshalton, Surrey

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In a previous paper (Aldridge & Cremer, 1955) it was concluded that triethyltin was an inhibitor of oxidative phosphorylation. The inhibitory properties of triethyltin differed from diethyltin which, like phenylarsenious acid, was primarily an inhibitor of α-oxo acid oxidases.

The classical inhibitor of oxidative phosphorylation is 2:4-dinitrophenol (Loomis & Lipmann, 1948). The formation of adenosine triphosphate may be prevented with little or no diminution of oxygen consumption. This behaviour is typical of a group of phenols such as 2:4:6-trichloro- (Parker, 1958), pentachloro- (Weinbach, 1954), *p*-nitro- (Terner, 1954) and 2:4-dinitro-phenols (Loomis & Lipmann, 1948). Since oxidation is not inhibited there is no difficulty in deciding that these substances do not act upon the respiratory chain but rather at some point between electron transport and the phosphorylation of adenosine diphosphate (Slater, 1953; Lardy, 1955). Recently, however, claims have been made that a heterogeneous group of substances are inhibitors of oxidative phosphorylation. Examples of these are chlorpromazine (Andrejew & Rosenberg, 1956; Abood, 1955), barbiturates (Brody & Bain, 1954), bilirubin (Zetterström & Ernster, 1956), thyroxine (Hoch & Lipmann, 1954) and its derivatives (Dickens & Salmony, 1956). All of these compounds, like triethyltin, inhibit oxidation as well as producing a depression of the phosphorylation quotient (P/O ratio).

Mitochondria prepared in different ways and studied in different media show various properties. For instance some preparations oxidize substrate at a rate which cannot be increased by the addition of enzymes utilizing adenosine triphosphate (Aldridge & Cremer, 1955). Mitochondria may now be prepared (Siekevitz & Potter, 1953; Maley & Lardy, 1955; Aldridge, 1957) with a low oxidative rate, which can be increased by the addition of systems utilizing adenosine triphosphate. The mitochondria used in this work have a low oxidative rate in an electrolyte medium at 37°, which may be increased three- to four-fold by the addition of apyrase, hexokinase and glucose or 2:4-dinitrophenol (Aldridge, 1957). Consideration of the two extremes of behaviour of different preparations of mitochondria indicate that it can be difficult to reach a firm conclusion that a substance, which decreases oxidation, is primarily an inhibitor of oxidative phosphorylation. On the one hand, when oxidation is rapid and not stimulated by apyrase or 2:4-dinitrophenol the mitochondria usually have high adenosine triphosphatase activity (Potter & Recknagel, 1951) thus giving low control P/O ratios. Under these conditions it is probable that an inhibitor of the respiratory chain will produce a lowering of the P/O ratio. On the other hand, if oxidation cannot proceed without the extramitochondrial utilization of adenosine triphosphate then it is difficult to understand why an inhibition of an energy-transferring step leading to the phosphorylation of adenosine diphosphate should not also produce the same inhibition of oxygen consumption. In view of these possibilities it was thought necessary to re-examine the effects of triethyltin and a series of homologues on oxidative phosphorylation. The results in this paper show that trialkyltins are inhibitors of a reaction which occurs between electron transport and before the formation of adenosine triphosphate. Many are highly active inhibitors, the most active being triethyltin, which can produce demonstrable effects at 10^{-7} M-concentration.

METHODS

Preparation of mitochondria. Mitochondria were isolated from rat liver, as described previously (Aldridge, 1957), except that a Potter–Elvehjem-type homogenizer, with a smooth glass tube and Perspex pestle with a total clearance of 0·02 in. instead of 0·03 in., was used.

Manometric technique. The reasons for the composition and the pH of the medium have been previously discussed (Aldridge, 1957). Each flask contained 3 ml. of a solution containing adenosine 5-phosphate (0·00115 M), adenosine triphosphate (ATP) (0·00106 M), KCl (0·1 M), $MgCl_2$ (0·014 M) ethylenediaminetetra-acetic acid (EDTA) (0·001 M), potassium phosphate (0·015 M), sucrose (0·025 M) and substrates [(0·01 M; except fumarate (0·001 M)]. For studies of oxidative phosphorylation each flask contained in addition glucose (180 μmoles), glycylglycine (50 μmoles) and hexokinase (200–400 units). This medium was adjusted to pH 6·7–6·8 with KOH, a glass electrode being

used, and all experiments were carried out at 37°. Carbon dioxide was absorbed by 0·15 ml. of 5M-KOH, the gas phase was air and 0·25–0·30 ml. of a mitochondrial suspension in 0·3 M-sucrose diluted to a volume (ml.) equal to twice the weight of the liver (g.) was used for all experiments.

Determinaton of adenosine triphosphatase activity. Each beaker contained 3 ml. of a solution containing ATP (0·003M), KCl (0·1M), $MgCl_2$ (0·014M), EDTA (0·001M) and sucrose (0·03M). The beakers were shaken at 37° in a Dubnoff metabolic shaking incubator. After temperature equilibration mitochondria were added and the mixture was incubated for 10 min. The reaction was stopped by the addition of perchloric acid and inorganic phosphate was determined.

Analytical methods. Inorganic phosphate was determined by the method of Fiske & Subbarow (1925). Protein was measured by the biuret method of Robinson & Hogden (1940) modified as described by Aldridge (1957). Mitochondrial protein has been expressed as mg. of albumin.

Inhibitors. Phenylarsine oxide was synthesized as follows: phenylarsonic acid was prepared (Vogel, 1948) followed by reduction by sodium bisulphite (A. H. Ford-Moore, personal communication). Assay by titration with iodine in the presence of bicarbonate indicated 99·7% of $C_6H_5 \cdot AsO$. The compound had an m.p. of 121–122° (uncorrected) in agreement with Nesmejanow & Freidlina (1934) (122°), but not with Blicke & Smith (1929) (144–146°). Diethyltin dichloride and all the trialkyltin acetates were supplied by Dr G. J. M. van der Kerk. These compounds were synthesized by published methods (van der Kerk & Luijten, 1956). Dimethylformamide is an excellent solvent for the trialkyltins, allowing stock solutions (usually 0·02M) to be prepared. A sample (0·03 ml.) of these solutions was added to each flask, final concentrations of 1% (v/v) dimethylformamide never being exceeded. This concentration has been found to produce a negligible effect upon oxidation and only a very slight stimulation of adenosine triphosphatase activity (Table 7).

2:4-Dinitrophenol (British Drug Houses Ltd.) was used from a stock solution (0·01M) neutralized to approx. pH 7.

Special chemicals and reagents. The following chemicals were obtained from the sources indicated: adenosine 5-phosphate, glycylglycine, sodium pyruvate, cocarboxylase (Roche Products Ltd.), disodium salt of ATP, coenzyme A, flavinadenine dinucleotide (Sigma Chemical Co., St Louis, Mo., U.S.A.), glucose, sodium fumarate (British Drug Houses Ltd.) and lipoic acid (L. Light and Co. Ltd.). All free acids were neutralized to approx. pH 6·8 with KOH before use.

Coenzyme I, cytochrome *c* and coenzyme II were prepared and assayed as previously described (Aldridge & Cremer, 1955; Aldridge, 1957). Potato apyrase was prepared by the method of Lee & Eiler (1951) and assayed by the method of Krishnan (1949); the preparation had an activity of 11 000 units/ml.

Hexokinase was prepared from baker's yeast by a modification by V. H. Parker (unpublished work) of the method of Berger, Slein Colowick & Cori (1946). The preparation was taken to the equivalent of their step 3*a* and when assayed by their procedure (but at 37° instead of 30°) had an activity of 3500 units/ml.

Units used. The oxidative activity of the mitochondria (q_{O_2}) is expressed as the μl. of O_2/mg. of protein/hr. Adenosine triphosphatase activity is given as μmoles of P liberated/mg. of protein/hr. The inhibitory power of substances is given as pI_{50} values, which is the negative logarithm of the molar concentration which will produce 50% inhibition.

RESULTS

Unstimulated and stimulated oxidation. In the usual mitochondrial preparation the rate of oxidation of a variety of substances must be regulated by the extra-mitochondrial utilization of the ATP formed (Potter & Recknagel, 1951; Siekevitz & Potter, 1953; Chance & Williams, 1955; Aldridge, 1957). The rate of oxidation of pyruvate by the present preparation of mitochondria is greatly increased by the addition of hexokinase and glucose, potato apyrase or 2:4-dinitrophenol (Aldridge, 1957). In the work to be described unstimulated oxidation (A) is the rate of oxygen uptake with no additions to the standard medium other than substrate, and stimulated oxidation (B) is the rate of oxygen uptake in the presence of apyrase, 2:4-dinitrophenol or hexokinase and glucose. The increase in oxygen uptake induced by these additions is the difference between the stimulated and unstimulated oxidation ($B-A$). The addition of apyrase brings about a three- to four-fold increase in the rate of oxygen uptake with pyruvate as substrate. Apyrase removes the terminal phosphorus of ATP 30 times faster than the other phosphorus atoms (Lee & Eiler, 1951) and the activity of apyrase in the medium used for the manometric experiments was 660 μg. atom of P liberated/ml./hr. Therefore the rate of dephosphorylation of ATP by the apyrase used (0·1 ml.) was 1·1 μg.atom/min. The mean rate of oxygen uptake in these experiments was 0·46 μg.atom/min. and the mean stimulation due to apyrase was 3·3-fold, indicating an oxygen uptake of 0·32 μg.atom/min. due to the addition of apyrase. A P/O ratio of 3 indicates a rate of phosphorylation of ADP of 0·96 μg.atom/min., a value in agreement with the rate of dephosphorylation by the apyrase. It is concluded therefore that the additional oxygen uptake in the presence of apyrase (i.e. oxidation induced by apyrase) is oxidation which is coupled to the phosphorylation of ADP. The unstimulated preparation which shows no adenosine triphosphatase activity (Table 7) nevertheless does oxidize pyruvate slowly, but whether the oxidative and energy-transferring processes involved are essentially the same as those in the stimulated preparation is not known. Since a large proportion (70–80%) of the total stimulated oxidation is oxidation induced by apyrase it has been possible to obtain accurate values for the inhibitory power (pI_{50}) of trialkyltins

Table 1. *Influence of trialkyltins on unstimulated and stimulated oxidation of pyruvate by mitochondria*

Mitochondria were added to the Warburg vessels containing the medium with or without apyrase (1100 units) and trialkyltins. After temperature equilibration readings were taken at 10 and 50 min. $pI_{50} = -\log$ M concentration of inhibitor to produce 50% inhibition, $pC = -\log$ M-concentration. These values were obtained graphically with results obtained with a range of concentrations forming a geometric progression with a common ratio of 4. The pI_{50} values for the oxidation induced by apyrase were calculated as described in the text and Figs. 1 and 2. The mean q_{O_2} for the unstimulated and stimulated oxidations were $26{\cdot}8 \pm 1{\cdot}5$ (10) and $85{\cdot}1 \pm 2{\cdot}9$ (10) respectively, indicating a mean 3·3-fold stimulation.

	Inhibition of O_2 uptake (pI_{50})			Stimulation of O_2 uptake by trialkyltins	
Compound	Unstimulated oxidation (A)	Stimulated oxidation (B)	Oxidation induced by apyrase ($B-A$)	Increase in rate of oxidation (q_{O_2})	Range of concentration of trialkyltin producing maximum stimulation (pC)
Trimethyltin	2·6	4·2	5·0	26–49	4·0–4·4
Triethyltin	4·2	5·5	6·6	30–55	5·5–5·8
Tri-*n*-propyltin	5·1	5·8	6·2	19–32	5·6–6·0
Tri-*iso*propyltin	5·1	5·7	6·3	36–45	5·7–6·0
Tri-*n*-butyltin	5·3	5·8	6·0	Nil	No stimulation
Tri-*n*-hexyltin	*	4·9	5·0	Nil	No stimulation
Tri-*n*-octyltin		No inhibition		Nil	No stimulation
Phenylarsenious acid	6·4	6·8	7·1	Nil	No stimulation
Diethyltin	4·6	4·9	5·1	Nil	No stimulation

* Less than 50% inhibition with a saturated solution.

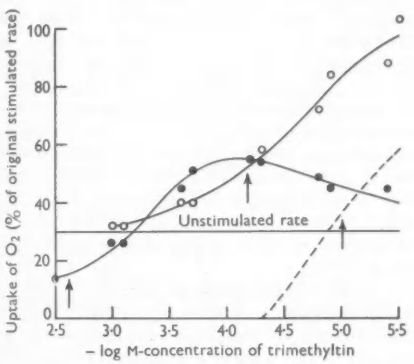

Fig. 1. Effect of trimethyltin on the stimulated and unstimulated oxidation of pyruvate by mitochondria. Each curve is derived from two experiments, each experiment contributing alternate points. ●, Unstimulated oxidation; ○ oxidation in the presence of apyrase (1100 units). Mitochondria were added to the Warburg flasks containing the medium and trimethyltin; readings were taken at 10 and 50 min. after placing the flasks in the bath at 37°. The broken line is the difference between the two curves. The pI_{50} (5·0) for the inhibition of the additional uptake of oxygen induced by apyrase has been derived from this line. (Arrows indicate the concentrations where oxidation is 50% inhibited.) q_{O_2} values for stimulated oxidation for the two experiments were 74 and 100.

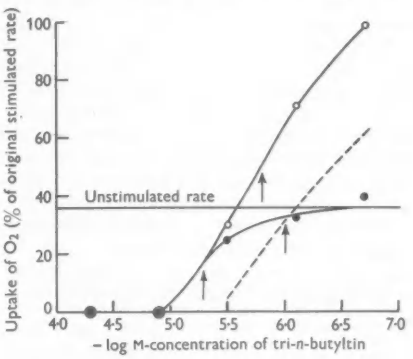

Fig. 2. Effect of tri-*n*-butyltin on the stimulated and unstimulated oxidation of pyruvate by mitochondria. ●, Unstimulated oxidation; ○, oxidation in the presence of apyrase (1100 units). Mitochondria were added to Warburg flasks containing the medium and tri-*n*-butyltin; readings were taken at 10 and 50 min. after placing the flasks in the bath. The broken line is the difference between the two solid curves. The pI_{50} (6·0) for the inhibition of the additional uptake of oxygen induced by apyrase has been derived from this line. (Arrows indicate the concentrations where oxidation is 50% inhibited.) q_{O_2} for the stimulated oxidation was 73.

against this induced oxidation, which is certainly coupled to phosphorylation. The method whereby these values have been derived from the experimental results is shown in Figs. 1 and 2. Triethyltin inhibits the oxidation of a variety of substrates by liver mitochondria (Aldridge & Cremer, 1955). The mitochondrial preparation used for that work must have been damaged since little or no increase in oxidation was obtained upon the addition of

hexokinase and glucose. With the present preparation it was found that the stimulated oxidation of pyruvate was prevented by lower concentrations of trialkyltin (pI_{50} 5·5, Table 1) than was originally found (pI_{50} approx. 4·7; Aldridge & Cremer, 1955). Examination of a series of trialkyltin compounds has shown that with the exception of tri-*n*-octyltin they inhibit both the unstimulated and the stimulated oxidation of pyruvate.

However, the unstimulated oxidation required a higher concentration of trialkyltin to produce a given inhibition. This is not a peculiarity of trialkyltins, for respiratory inhibitors such as phenylarsenious acid (Peters, 1955) and diethyltin (Aldridge & Cremer, 1955) behave similarly. The difference in the concentrations required to inhibit 50% of the unstimulated and stimulated oxidation is largest with trimethyl and decreases in the order trimethyl, triethyl, tripropyl until at tri-*n*-butyltin it is approximately the same as the difference found for phenylarsenious acid and diethyltin.

During the examination of the sensitivity to trialkyltins of the unstimulated oxidation it was observed that the lower homologues produced considerable stimulation of oxidation. In Fig. 1 the behaviour of trimethyltin is illustrated. Over a considerable range of concentration an increase in oxygen uptake was observed. Trimethyltin and triethyltin produced comparable stimulation but the extent of the stimulation decreased with tri-*n*-propyl and tri-*iso*propyl until with tri-*n*-butyl and higher homologues it was undetectable (Table 1). Fig. 2 shows the behaviour of tri-*n*-butyltin, which is typical of the higher homologues.

Triethyl-, tri-*n*-propyl-, tri-*iso*propyl- and tri-*n*-butyl-tin all inhibit stimulated oxidation at approx. 10^{-6} M. There is a decrease in activity on either side of this series, trimethyl and tri-*n*-hexyl being less active and tri-*n*-octyl being completely inactive, owing perhaps to its insolubility in water.

Since evidence has been produced that triethyltin is an inhibitor of oxidative phosphorylation (Aldridge & Cremer, 1955), it was important to obtain a value for the inhibitory power of the trialkyltins against oxidation which was coupled to phosphorylation. The pI_{50} values for the inhibitory action of trialkyltins upon this oxidation have been derived as shown in Figs. 1 and 2 and are listed in Table 1. It is clear that oxidation which is coupled to phosphorylation is very sensitive to trialkyltins, the concentration of triethyltin producing 50% inhibition being $2{\cdot}5 \times 10^{-7}$ M. With a preparation of mitochondria which would oxidize succinate at a similar rate with or without the addition of nucleotide, succinate oxidation has previously been shown to be unaffected by triethyltin (Aldridge & Cremer, 1955). This oxidation of succinate must have been unaccompanied by phosphorylation of nucleotide. Oxidation of succinate by the present preparation was at least partially coupled to phosphorylation for the unstimulated oxidation was stimulated two- or three-fold by the addition of apyrase (Aldridge, 1957). It was interesting to find that the oxidation of succinate had now become sensitive to triethyltin and inhibition of succinate oxidation induced by apyrase was obtained at the same concentrations which inhibit induced pyruvate oxidation (Tables 1, 2). Also the unstimulated oxidation of succinate was increased by triethyltin but only a slight stimulation was produced by tri-*n*-butyltin.

Oxidative phosphorylation. With the system for the measurement of oxidative phosphorylation, with an excess of hexokinase and glucose in order to trap any ATP formed, trialkyltins are potent inhibitors of oxidation and phosphorylation. With all the trialkyltins phosphorylation was inhibited more than oxidation with a given concentration of inhibitor. This was not so with the respiratory inhibitor phenylarsenious acid (Table 3 and Aldridge, 1957). It is clear, however, that the difference between the inhibition of oxygen and phosphate uptake is not great (16–26%) and does not approach that obtained with 2:4-dinitrophenol. The P/O ratios were much reduced when oxygen uptake was 50% inhibited by trialkyltins. It is, however, important to appreciate that P/O ratios give an exaggerated impression of the divergence of the inhibition of uptakes of oxygen and phosphorus determined experimentally. It is interesting to note that the pI_{50} values for trialkyltins against oxidation in the presence of a large excess of hexokinase agreed closely with those for the oxidation induced by apyrase (Table 6).

Table 2. *Influence of trialkyltin on the oxidation of succinate by mitochondria*

Mitochondria were added to the Warburg vessels containing the medium with or without apyrase (1100 units) and trialkyltin. Readings were taken at 10 and 40 min. after placing the vessels in the bath. $pI_{50} = -\log$ M-concentration of inhibitor to produce 50% inhibition. These values were obtained graphically with results obtained with a range of five concentrations forming a geometric progression with a common ratio for triethyltin of 4 and for tri-*n*-butyltin of 3. The pI_{50} values for the oxidation induced by apyrase were calculated as described in the text and Figs. 1 and 2. The mean q_{O_2} for the unstimulated and stimulated oxidation were 47 and 122, indicating a mean 2·6-fold stimulation.

	Inhibition of oxidation (pI_{50})		
Trialkyltin	Unstimulated oxidation	Stimulated oxidation	Oxidation induced by apyrase
Ethyl	4·2	5·0	6·8
n-Butyl	5·1	5·5	5·9

Table 3. *Influence of trialkyltin compounds on oxidation of pyruvate and coupled phosphorylation*

Mitochondria were added to the Warburg vessels containing the medium and trialkyltin. After temperature equilibration readings were taken at 10 min., 13 min. 20 sec., 16 min. 40 sec. and 20 min. The rate of uptake of oxygen was calculated from the slope of the best straight line through these points. At 22 min. 0·5 ml. of 60% $HClO_4$ was added to each flask. To a control set of flasks 0·5 ml. of 60% $HClO_4$ was added at 10 min. pI_{50} values ($-\log$ M-concentration of inhibitor to produce 50% inhibition) were obtained graphically with the results obtained with a range of concentrations forming a geometric progression with a common ratio of 2. The inhibition of uptake of phosphorus (%) and the P/O ratios when uptake of oxygen was 50% inhibited were derived from these graphs.

Compound	Control (q_{O_2})	pI_{50}		Control P/O ratio	P/O ratio when O_2 uptake was 50% inhibited	% Inhibition of P uptake when O_2 uptake was 50% inhibited
		O_2 uptake	P uptake			
Trimethyltin	95	5·0	5·4	2·4	1·2	76
Triethyltin	77	6·8	7·1	2·6	1·8	66
Tri-*n*-propyltin	88	6·2	6·5	2·9	1·6	72
Tri-*iso*propyltin	100	6·1	6·2	2·8	1·5	73
Tri-*n*-butyltin	98	6·0	6·2	2·7	1·4	74
Tri-*n*-hexyltin	97	5·5	5·6	2·8	1·8	68
Tri-*n*-octyltin	76	—	—	2·9	—	—
Phenylarsenious acid	104	6·9	6·9	2·8	2·6	54
2:4-Dinitrophenol	109	—	5·1	2·8	0·1*	92†

* P/O ratio when O_2 uptake was 17% inhibited.
† Inhibition of P uptake (%) when O_2 uptake was 17% inhibited.

Table 4. *Influence of coenzymes and trialkyltin on oxidation of pyruvate and coupled phosphorylation*

Cofactors [500 μg. of cytochrome *c*, 100 μg. of coenzyme I (Co I) and coenzyme II (Co II) and 10 μg. of coenzyme A (Co A), cocarboxylase, lipoic acid and flavinadenine dinucleotide (FAD)] were added to the flask when required. The triethyltin added is given below. Mitochondria were added last. After equilibration readings were taken at 10, 14, 18, 22 and 26 min. At 7, 11, 15, 19, 23 and 27 min. 0·5 ml. of 60% $HClO_4$ was added to a flask from each series and the concentration of inorganic P was determined. The rate of O_2 uptake and of P uptake was calculated from the slope of the best straight line through these points.

Concentration of triethyltin (M)	q_{O_2}	O_2 uptake (μg.atom/min.)	P uptake (μg.atom/min.)	P/O
No coenzymes added				
Nil	99	0·475	1·355	2·85
$3{\cdot}12 \times 10^{-7}$	49	0·234	0·37	1·58
Co I, Co II, FAD, cytochrome *c* added				
Nil	100	0·362	1·05	2·91
$3{\cdot}12 \times 10^{-7}$	69	0·25	0·43	1·72
Co I, Co II, FAD, cytochrome *c*, cocarboxylase, lipoic acid, Co A added				
Nil	119	0·80	2·40	3·00
$3{\cdot}12 \times 10^{-7}$	86	0·573	1·03	1·80

The possibility that the inhibition of oxidation was due to a loss of essential respiratory coenzymes was tested. The results in Table 4 show that although the addition of a variety of coenzymes slightly influenced the inhibition of oxidation, it has not influenced the lowering of phosphate uptake produced by the triethyltin. Parallel experiments with succinate as substrate gave similar results.

Oxidation stimulated by apyrase, hexokinase and 2:4-dinitrophenol

Oxidation stimulated by hexokinase and glucose (Table 3) is more sensitive to trimethyl and triethyltin than oxidation stimulated by apyrase (Table 1): for instance, the pI_{50} values for trimethyltin are 5·0 and 4·2 and for triethyltin 6·8 and 5·5 respectively. Consideration of the experiments showed that conditions were not identical. Hexokinase was added in large excess, in order to trap all ATP formed, whereas only sufficient apyrase was added to produce about the maximum rate of oxidation. In fact 14 units of hexokinase were sufficient to produce maximum rate of oxidation, whereas 350 units were added in the oxidative phosphorylation experiments. In Table 5 is shown a comparison of the inhibitory power of triethyltin upon oxidation stimulated by the minimal amounts

of apyrase, 2:4-dinitrophenol and hexokinase with oxidation stimulated by a large excess of hexokinase. The last system is the most sensitive to triethyltin and the others are all of the same sensitivity.

Oxidation stimulated by 2:4-dinitrophenol and by apyrase is equally inhibited by other trialkyltins (Table 6). Neither apyrase nor hexokinase was inhibited by the concentrations of trialkyltins used, and since the concentrations of 2:4-dinitrophenol were very much higher than the effective concentrations of many of the trialkyltins, any reaction of trialkyltins with 2:4-dinitrophenol cannot explain these results.

Mitochondrial adenosine triphosphatase. Since some trialkyltins, like 2:4-dinitrophenol, stimulate oxidation their influence upon the latent adenosine triphosphatase of liver mitochondria was examined. These mitochondria have a negligible adenosine

Table 5. *Inhibition by triethyltin of oxidation of pyruvate stimulated by various agents*

Mitochondria were added to the Warburg flasks containing the medium, triethyltin and apyrase (1100 units) or 2:4-dinitrophenol (2×10^{-5} M) or hexokinase (14 or 350 units). Readings were taken at 5 min. intervals from 10 to 40 min. after placing in the bath. Rate of uptake of oxygen was calculated from the best straight line through these points. q_{O_2} values: for unstimulated control, 20; for oxidation stimulated by apyrase, 72; for 2:4-dinitrophenol 71; for 14 units of hexokinase, 74, and for 350 units of hexokinase, 76.

	O_2 uptake (% of control)			
Concn. of triethyltin (M)	Hexokinase (350 units)	Hexokinase (14 units)	Apyrase	2:4-Dinitrophenol
$8{\cdot}65 \times 10^{-7}$	27	51	55	59
$2{\cdot}88 \times 10^{-7}$	49	68	74	—
$9{\cdot}6 \times 10^{-8}$	78	81	88	81

Table 6. *Inhibitory power of trialkyltins against oxidation stimulated by apyrase, 2:4-dinitrophenol and hexokinase and against 2:4-dinitrophenol-activated adenosine triphosphatase*

Oxidation was stimulated by 10^{-5} M-2:4-dinitrophenol. pI_{50} values were calculated for oxidation induced by 2:4-dinitrophenol as shown in Figs. 1 and 2. Other pI_{50} values are taken from Tables 1, 3 and 7.

	pI_{50}			
Trialkyltin	Oxidation induced by apyrase	Oxidation induced by 2:4-dinitrophenol	Oxidation in the presence of excess of hexokinase	2:4-Dinitrophenol-activated adenosine triphosphatase
Methyl	5·0	5·1	5·0	5·3
Ethyl	6·6	6·5	6·8	6·7
n-Propyl	6·2	6·1	6·2	6·4
*iso*Propyl	6·3	6·1	6·1	6·3
n-Butyl	6·0	5·9	6·0	6·1
n-Hexyl	5·0	4·8	5·5	4·5
n-Octyl	—	—	—	—

Table 7. *Action of trialkyltins on latent and 2:4-dinitrophenol-activated adenosine triphosphatase*

Liberation of inorganic phosphate was determined 10 min. after the addition of mitochondria to the medium containing trialkyltin. The adenosine triphosphatase (ATPase) activity of the mitochondria alone was negligible and in the presence of 1% (v/v) dimethylformamide and 10^{-5} M-2:4-dinitrophenol was $0{\cdot}35 \pm 0{\cdot}06$ (10) and $9{\cdot}0 \pm 0{\cdot}16$ (13) μmoles of P/mg. of protein/hr. respectively. Activity in the presence of dimethylformamide has been subtracted from the results. pI_{50} values were corrected for the activation of ATPase by trialkyltins as shown in Figs. 3 and 4.

	Activation of ATPase		Inhibition of ATPase activated by 10^{-5} M-2:4-dinitrophenol	
Compound	Range of concn. producing maximum activation (pC)	Maximum activation produced (μmoles of P/mg. of protein/hr.)	pI_{50}	pI_{50} (corrected for stimulation of ATPase by trialkyltin)
Trimethyltin	4·1–4·6	5·1	3·8	5·3
Triethyltin	5·7–6·1	4·0	5·9	6·7
Tri-*n*-propyltin	6·1–6·3	2·9	6·3	6·4
Tri-*iso*propyltin	6·0–6·2	1·8	6·2	6·3
Tri-*n*-butyltin	5·8–5·9	1·6	6·1	6·2
Tri-*n*-hexyltin	*	*	4·3	4·5
Tri-*n*-octyltin	—	—	—	—

* ATPase activated at concentrations when tri-*n*-hexyltin was not all in solution.

triphosphatase activity in an electrolyte medium containing magnesium (cf. Lardy & Wellman, 1953; Potter & Recknagel, 1951). Dimethylformamide, the solvent used for the trialkyltins, induced a low, and $3{\cdot}3 \times 10^{-5}$ M-2:4-dinitrophenol a high activity (0·3 and 13·9 μmoles of P/μg. of protein/hr. respectively). With the exception of tri-*n*-octyltin all the trialkyltins tested produced some activation of adenosine triphosphatase. The largest activation was produced by trimethyl- and triethyl-tins but even with these compounds it was only one-third of the maximum activation of adenosine triphosphatase in the presence of $3{\cdot}3 \times 10^{-5}$ M-2:4-dinitrophenol or one-half of the activity with 10^{-5} M. Only those trialkyltins which stimulated oxidation also activated adenosine triphosphatase appreciably, and the order of the activity of the trialkyltins in the two systems is the same (Tables 1 and 7). The concentrations which give maximum activation of adenosine triphosphatase are slightly lower than those producing maximum stimulation of oxidation (Tables 1 and 7). It should be noted that tri-*n*-butyltin activated adenosine triphosphatase to a small extent, whereas no stimulation of oxidation was obtained. However, a small stimulation of oxidation would have been very difficult to detect in these experiments.

Activation of adenosine triphosphatase is also obtained with higher concentrations of tri-*n*-butyltin (Fig. 4) and this also occurs with tri-*n*-propyl-, tri-*iso*propyl- and tri-*n*-hexyl-tins at similar concentrations.

Trialkyltins inhibit oxidation stimulated by apyrase, hexokinase and glucose or 2:4-dinitrophenol (Table 6). The influence of trialkyltins upon adenosine triphosphatase stimulated by 2:4-dinitrophenol was therefore examined. It was found that all the trialkyltins with the exception of tri-*n*-octyltin prevent the activation of adenosine triphosphatase by 2:4-dinitrophenol (Table 7). The relation between concentrations of inhibitor and inhibition for triethyltin and tri-*n*-butyltin is shown in Figs. 3 and 4. The concentration where the activation by 10^{-5} M-2:4-dinitrophenol is 50% of its maximum has been calculated (cf. Figs. 3 and 4 for method of calculation) and the pI_{50} values are listed in Table 7. Since 10^{-5} M-2:4-dinitrophenol was used for the stimulation of oxidation for the purposes of comparison this concentration was used for these experiments upon adenosine triphosphatase. Inhibitors of respiration such as cyanide, phenylarsenious acid or diethyltin did not prevent the activation of adenosine triphosphatase by 2:4-dinitrophenol (Table 7).

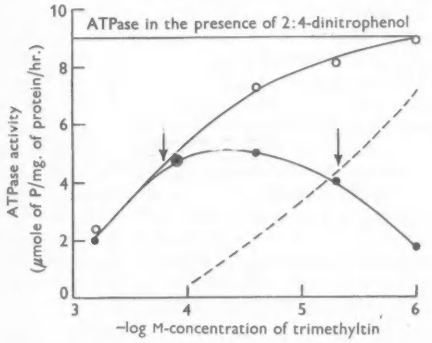

Fig. 3. Influence of trimethyltin on latent and 2:4-dinitrophenol-activated adenosine triphosphatase (ATPase). ●, Latent ATPase; ○, ATPase activated by 10^{-5} M-2:4-dinitrophenol. Mitochondria were added to the medium containing trimethyltin with or without 2:4-dinitrophenol. After 10 min. 0·5 ml. of 60% $HClO_4$ was added and the liberated phosphate determined. Control activity in the presence of 1% (v/v) dimethylformamide was 0·26 μmole of P/mg. of protein/hr. The broken line is the difference between the two solid curves. The pI_{50} for the inhibition by trimethyltin of the ATPase activated by 2:4-dinitrophenol (uncomplicated by ATPase activation by trimethyltin) has been derived from this line. Arrows indicate the concentration where ATPase is 50% inhibited.

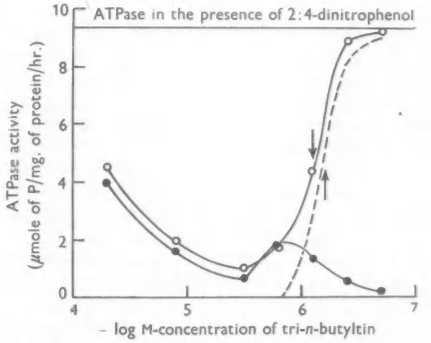

Fig. 4. Influence of tri-*n*-butyltin on latent and 2:4-dinitrophenol-activated adenosine triphosphatase (ATPase). ●, Latent ATPase; ○, ATPase activated by 10^{-5} M-2:4-dinitrophenol. Mitochondria were added to the medium containing tri-*n*-butyltin or tri-*n*-butyltin and 2:4-dinitrophenol. After 10 min. 0·5 ml. of 60% $HClO_4$ was added and the liberated phosphate determined. Control activity in the presence of 1% (v/v) dimethylformamide was 0·38 μmole of P/mg. of protein/hr. The broken line is the difference between the two solid curves. The pI_{50} for the inhibition by tri-*n*-butyltin of the ATPase activated by 2:4-dinitrophenol (uncomplicated by ATPase activation by tri-*n*-butyltin) has been calculated from this line. Arrows indicate the concentration where ATPase is 50% inhibited.

DISCUSSION

Six of the seven trialkyltin compounds tested are inhibitors of the oxidation of pyruvate by liver mitochondria. Some of the lower molecular-weight homologues also activate the adenosine triphosphatase of mitochondria. The question to be decided is whether the inhibition of oxidation is secondary to a primary inhibition of phosphorylation or whether trialkyltins have a dual action, one stimulating adenosine triphosphatase and the other inhibiting electron transport, their final actions being a combination of these two.

There are several reasons why the inhibition of electron transport is unlikely. Previous work (Aldridge & Cremer, 1955) indicated that in some preparations of mitochondria neither succinate oxidase nor diphosphopyridine nucleotide–cytochrome *c* reductase is inhibited by triethyltin. Assuming that in these mitochondria electron transport flows through the same respiratory chain as in other preparations in which oxidation is coupled to phosphorylation, these results would indicate that electron transport is not inhibited by triethyltin. In the presence of excess of hexokinase for a given inhibition of oxygen uptake, a similar inhibition of phosphate uptake and lowering of P/O ratio is obtained for all the trialkyltins (Table 3). Only trimethyl- and triethyl-tin produce an appreciable activation of adenosine triphosphatase. If the enzyme were an important factor in the apparent inhibition of phosphorylation by trialkyltins a larger difference would be found for the lower members. Oxidation is most sensitive to trialkyltins when nearly all the uptake of oxygen is being utilized for the esterification of adenosine diphosphate (i.e. in the presence of a large excess of hexokinase and a P/O ratio of 2·7 or more). When maximum oxygen uptake is obtained by the addition of minimal amounts of apyrase, hexokinase or 2:4-dinitrophenol oxidation is less inhibited. This is particularly so for trimethyl- and triethyl-tin and is probably associated with the liberation of adenosine triphosphatase activity by these compounds. Oxidation therefore can occur in the presence of concentrations of trialkyltins higher than those known to prevent phosphate esterification. Inhibitors of the respiratory chain like cyanide, phenylarsenious acid and diethyltin do not prevent the activation of mitochondrial adenosine triphosphatase by 2:4-dinitrophenol. Trialkyltins on the contrary are potent inhibitors of adenosine triphosphatase activated by 2:4-dinitrophenol or prevent the activation of it by 2:4-dinitrophenol. Therefore for a variety of reasons it seems impossible to formulate a hypothesis which will explain the biochemical properties of the trialkyltins in terms of a dual action involving electron transport and adenosine triphosphatase.

An alternative hypothesis, that the primary block is in the energy-trapping and -transferring reactions between electron transport and the formation of ATP, is one which fits the present experimental findings. Preparations of mitochondria in which oxidation and the phosphorylation of ATP are coupled are very sensitive to trialkyltins. Succinate oxidation, when it can be stimulated by apyrase and is therefore utilized in the phosphorylation of adenosine diphosphate, is inhibited by trialkyltins. There is good agreement between the concentrations of trialkyltin required to inhibit oxidation induced by apyrase, 2:4-dinitrophenol or hexokinase, oxidation in the presence of excess of hexokinase and adenosine triphosphatase activated by 2:4-dinitrophenol (Table 6). The agreement covers the seven trialkyltins studied. There seems no doubt that 2:4-dinitrophenol acts at some stage between electron transport and the formation of ATP. It has recently been shown in this Laboratory that there is a good correlation between the concentrations necessary for the stimulation of oxidation and the activation of adenosine triphosphatase by 2:4:6-trichloro-, pentachloro-, *p*-nitro- and 2:4-dinitro-phenols (Parker, 1958). Both phenomena therefore seem to be due to the same primary action. There is good agreement between the concentration of trialkyltins necessary to inhibit the forward (oxidation) and the back (adenosine triphosphatase) reactions induced by 2:4-dinitrophenol. It is therefore probable that trialkyltins are inhibiting the same step in the process studied in the different directions. A consequence of this conclusion is that the trialkyltins are blocking at the stage where 2:4-dinitrophenol acts. It must be emphasized that it is not suggested that 2:4-dinitrophenol and trialkyltin have the same mechanism of action. But if it may be accepted that trialkyltins inhibit a specific enzyme then it follows from these results that the mechanism of action of 2:4-dinitrophenol also involves this enzyme and is not a simple chemical reaction with a 'high-energy' intermediate (unless this is the enzyme). Rather 2:4-dinitrophenol may be acting as an acceptor for a group transferred by an enzyme and trialkyltins block this enzymic step. Hypothetical series of reactions have been postulated for the trapping of the energy of oxidation of reduced coenzymes (Slater, 1953; Lardy, 1955; Cooper & Lehninger, 1957; Chance & Hollunger, 1957). Since the nature of these reactions is at present unknown it is not possible to draw any conclusions about the stage at which a trialkyltin or 2:4-dinitrophenol acts.

In the above discussion the activation of adenosine triphosphatase by some trialkyltins is

not implicated in the primary mode of action of trialkyltins. Indeed the fact that activation of adenosine triphosphatase always occurs at concentrations higher than those necessary to prevent phosphate esterification in respiring mitochondria indicates that it is a different phenomenon. As shown in Fig. 4, as the concentration of tri-*n*-butyltin is increased from 10^{-7} M, adenosine triphosphatase is slightly activated at 10^{-6} M. As the concentration is further increased, a decrease in adenosine triphosphatase activity is followed by an appreciable activation. These results indicate that there are two types of adenosine triphosphatase activation. As pointed out, the stimulation of oxidation by trialkyltins parallels their ability to stimulate adenosine triphosphatase, but this correlation applies only to the stimulation at the lower concentrations of trialkyltins. Trimethyl- is the most active, followed in decreasing order by triethyl-, tri-*n*-propyl-, tri-*iso*propyl- and tri-*n*-butyl-tins. Fat-soluble substances modify mitochondrial membranes (Christie & Judah, 1954) and the trialkyltins are more soluble in organic solvents than in water. However, the most active member of the series is trimethyltin, the least soluble in organic solvents. It seems unlikely, therefore, that the adenosine triphosphatase accompanied by stimulation of oxidation is caused by structural damage to the mitochondria. The other type of activation of adenosine triphosphatase occurs at higher concentrations (10^{-4} M) of tri-*n*-propyl-, tri-*iso*propyl-, tri-*n*-butyl- and tri-*n*-hexyl-tins. At these concentrations oxidation is completely inhibited, and since these compounds are the more fat soluble it is possible that this is a structural damage to the mitochondria.

Of the trialkyltins examined only tri-*n*-octyltin is inactive. Experience with tri-*n*-hexyltin has shown that it is less consistent in its behaviour than the lower homologues. The behaviour of these higher molecular-weight homologues is probably due to their low solubility in water. The other trialkyltins are all very active and extremely consistent in their behaviour. Triethyltin is the most active (10^{-7} M) and is undoubtedly the most active inhibitor of oxidative phosphorylation known.

The results in Table 3 show that the difference between the inhibition of uptakes of oxygen and phosphate are not large. Theoretically if mitochondrial oxidation cannot occur without the utilization of ATP then inactivation of any enzymic step in this process must lead to the same lowering of uptake of oxygen and phosphate and must give a constant P/O ratio. If it is accepted that such a relationship between oxidation and phosphorylation exists in normal mitochondria then inhibitors such as the trialkyltins cannot be expected to produce profound lowering of P/O ratios. Indeed if the experiments were perfect no change should be found. With damaged mitochondria (possessing free adenosine triphosphatase activity) a lowering of P/O ratio is more likely, but then even a respiratory inhibitor may give such a result. For this reason it is important to use a standard respiratory inhibitor to check the constancy of the P/O ratio under conditions of lowered oxygen uptake with the particular preparation of mitochondria used. Phenylarsenious acid (Peters, 1955) is very suitable for this purpose. It is highly active and appears to be a specific inhibitor of the oxidation of α-oxo acids, for under all the conditions examined an inhibition of oxygen uptake is always accompanied by an accumulation of α-oxo acids. For up to 70% inhibition of oxygen uptake no change in the P/O ratio is obtained.

In the following paper (Stoner & Threlfall, 1958) attempts to apply these findings *in vitro* to the study of the toxicity of the trialkyltin compounds in the whole animal are described. The results show the difficulty of correlating findings *in vitro* and findings *in vivo*, for in the whole animal no unequivocal evidence was found with the techniques now available for a derangement of oxidative phosphorylation by triethyltin, and it would have been impossible to deduce that this was its action *in vitro*.

SUMMARY

1. Rat-liver mitochondria whose rate of oxidation of pyruvate may be increased three- to four-fold by the addition of potato apyrase, hexokinase and glucose and 2:4-dinitrophenol have been used to examine the biochemistry of trialkyltins.

2. Oxidation induced by apyrase, 2:4-dinitrophenol or hexokinase and glucose, oxidation in the presence of excess of hexokinase and glucose and adenosine triphosphatase stimulated by 2:4-dinitrophenol are all equally sensitive to inhibition by a trialkyltin. Seven trialkyltins have been tested against these processes.

3. The lower molecular-weight trialkyltins induce some adenosine triphosphatase activity in mitochondria and this has been correlated with a stimulation of the oxidation of pyruvate.

4. It is concluded that trialkyltins inhibit a step in the energy-transferring chain between electron transport and the formation of adenosine triphosphate.

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The Biochemistry of Organotin Compounds

EFFECT OF TRIETHYLTIN SULPHATE ON TISSUE PHOSPHATES IN THE RAT

By H. B. STONER and C. J. THRELFALL
Unit for Research in Toxicology, M.R.C. Laboratories, Carshalton, Surrey

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In the preceding paper Aldridge (1958) has shown that trialkyltin compounds interfere with the process of oxidative phosphorylation in liver mitochondria and he has also shown (personal communication) that brain mitochondria are equally sensitive. The toxicity of these compounds to the whole animal has been known for a long time (Jolyet & Cahours, 1869) and has recently been reinvestigated in this Laboratory (Stoner, Barnes & Duff, 1955; Barnes & Stoner, 1958). The parallel investigation of these compounds at different levels of biological organization has led to an attempt, so far unsuccessful, to explain the toxicity in biochemical terms. The main effect of these compounds *in vivo* is upon the central nervous system, which is also the site of the only significant pathological change, an interstitial oedema confined to the white matter of the brain and spinal cord (Magee, Stoner & Barnes, 1957). Difficulties in elucidating the biochemical mechanisms of their toxicity have already been encountered. Although liver and brain mitochondria are equally affected *in vitro*, Cremer (1957), working with tissue slices, found that it was only the uptake of oxygen by slices of nervous tissue which was inhibited whether the triethyltin was added *in vitro* or injected into the rat before it was killed. This was despite the fact that after the injection of triethyltin there was more in the liver than brain and that the temperature of both was depressed after the injection.

Triethyltin is the most active member of the series *in vitro* and *in vivo* and its effects on some phosphate components of rat tissues and on the distribution of injected ^{32}P has now been studied in the whole animal. Triethyltin affects the metabolism of both the nervous system and liver in the rat but the mechanism of the derangement cannot yet be deduced from these results nor can they be readily correlated with the previous findings *in vitro*.

METHODS

Male albino rats of the Porton strain (145±10 g. body wt.) were used. They were fed either on M.R.C. Diet 41 (Bruce & Parkes, 1949) or more recently on Diet 41B (Bruce & Parkes, 1956) given *ad lib.* In the experiments on nephrectomized rats both kidneys were removed through a ventral

incision under ether anaesthesia on the afternoon before the experiment.

Larger rats (250–300 g.) had to be used for the measurement of tissue temperatures. The brain temperature was measured with copper–constantan (Eureka) thermocouples (36 s.w.g.) inserted into the brain under ether anaesthesia on the day before the experiment. The liver temperature was measured and its blood flow assessed by the internal calorimetry method of Grayson (1952), used as described previously (Stoner, 1956) except that the rats were not given oxytetracycline hydrochloride.

Triethyltin sulphate was prepared by Dr G. J. M. van der Kerk (Organisch Chemisch Institut, T.N.O., Utrecht, Holland). For injection it was dissolved in 0·9% NaCl and neutralized to give a solution of 5 mg./ml. The controls received an equivalent volume of 0·9% NaCl.

Radioactive phosphate (^{32}P) was obtained from the Radiochemical Centre, Amersham, Bucks, as a solution in sterile isotonic phosphate–buffer (pH 7; 1 mg. of P/ml.). It was assayed before use as described by Stoner, Threlfall & Green (1952) and injected intramuscularly in doses of either 25 or 50 μc/100 g. body wt.

At intervals after the injection of triethyltin or 0·9% NaCl and ^{32}P the rats were anaesthetized with pentobarbitone sodium (Veterinary Nembutal; Abbott Laboratories Ltd.) given intraperitoneally (5 mg./100 g. body wt.) and the tissues sampled in two ways.

As a routine procedure the chest was opened and a sample of blood collected with a Pasteur pipette after division of the great vessels. Heparin (British Drug Houses Ltd.) was used as an anticoagulant and the plasma was obtained by centrifuging the blood immediately in a plastic tube. The plasma inorganic P was determined after precipitation as $MgNH_4PO_4$ from a trichloroacetic acid extract of the plasma (Stoner *et al.* 1952). A sample of liver was taken and the brain and spinal cord were dissected out as quickly as possible. Trichloroacetic acid extracts of these tissues were prepared as described by Webster (1954). The precipitate was washed once with 10% (w/v) trichloroacetic acid and twice with water. Lipid P was then extracted by the method of Davidson, Frazer & Hutchison (1951). The final residue was dried and ashed as described by Webster (1954). The P content of these fractions was determined, in duplicate, by the method of Fiske & Subbarow (1925). Their radioactivity was determined on the same solutions with an M6 liquid counter (20th Century Electronics Ltd.) attached to conventional scaling apparatus, and the usual corrections were made.

The following modifications were adopted for the fractionation of the acid-soluble P fraction. For the brain the anaesthetized rat was plunged in liquid N_2, the cerebral hemispheres were dissected out when the rat was frozen and the tissue was stored in liquid N_2. For the liver, a sample was quickly removed from the anaesthetized rat and frozen in liquid N_2.

The liver and brain nucleotides were separated as described by Threlfall (1957) with 6 vol. and 12 vol. of 3% (v/v) perchloric acid respectively for extraction and 150 mg. and 100 mg. of Norit SX30 special charcoal (Haller and Phillips, Ltd. London) for adsorption. Separation was achieved by two-dimensional paper chromatography with the *iso*butyric acid–aq. NH_3 soln. and the acetone–water systems as a routine procedure. This combination of systems ensured the complete separation of the inorganic P from the nucleotide components necessary for determinations of radioactivity. P was estimated by the method of Berenblum & Chain (1938) as previously described (Threlfall, 1957) and the radioactivity of the *iso*butanol was determined as above, after the P estimation. The total acid-soluble adenine content of the liver was assessed from the ultraviolet-light-absorption spectrum of suitably diluted perchloric acid extracts.

The blood content of the liver was determined with ^{32}P-labelled erythrocytes (Chaplin, 1954). A small volume (0·25 ml.) of labelled blood was injected into a tail vein of a rat under pentabarbitone anaesthesia; after equilibration for about 10 min., samples of blood and liver were taken, extracted with 3% (v/v) perchloric acid and their radioactivity was determined.

The water content of the tissues was determined by drying to a constant weight at 105°. The liver was examined histologically in paraffin sections stained with haematoxylin and eosin after fixation in Helly's fluid. Some of the rats used for histological studies were given 0·07 mg. of colchicine/100 g. body wt. just before the start of the experiment and 4 hr. before the rat was killed.

Results are expressed as the mean $\pm$ standard deviation and have been examined statistically by Student's t test as modified by Fisher (1934) for small samples.

The following abbreviations have been used: adenosine mono-, di- and tri-phosphate, AMP, ADP and ATP; di- and tri-phosphopyridine nucleotide, DPN and TPN.

RESULTS

Toxicity of triethyltin

The standard dose of triethyltin sulphate used in these experiments was 1·2 mg./100 g. body wt., injected intraperitoneally. This dose is two to three times the LD_{50} and is always fatal, the survival time being about 48 hr. Signs of poisoning appear about 10 min. after the injection. The hind limbs become weak and flaccid and the rat has difficulty in walking. The rat quickly becomes prostrate. After a few hours there is some recovery but this is never complete and is soon replaced by progressive muscular weakness. After 24 hr. the rat usually appears moribund. The present experiments were confined to the first 24 hr. of the illness. The typical changes in the temperature of the brain and liver and in the hepatic blood flow during this period are shown in Fig. 1. Although triethyltin is not rapidly excreted in the urine, bilateral nephrectomy shortened the survival time so that many of these rats given the standard dose did not survive 24 hr. A lower dose was used in some of these experiments.

Effect of triethyltin on the plasma inorganic phosphate level

In normal rats the plasma inorganic P level rose significantly after the administration of triethyltin (Table 1). After nephrectomy this inorganic P level was high and the difference between the

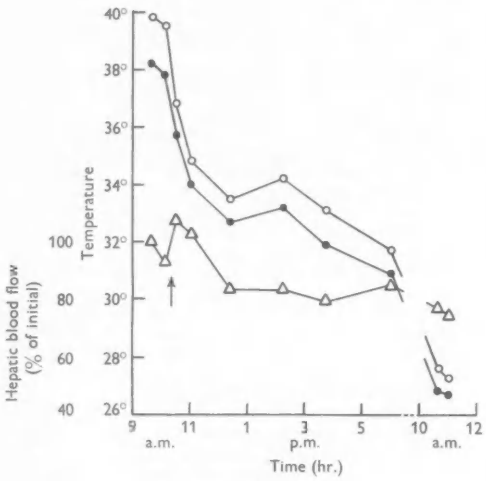

Fig 1

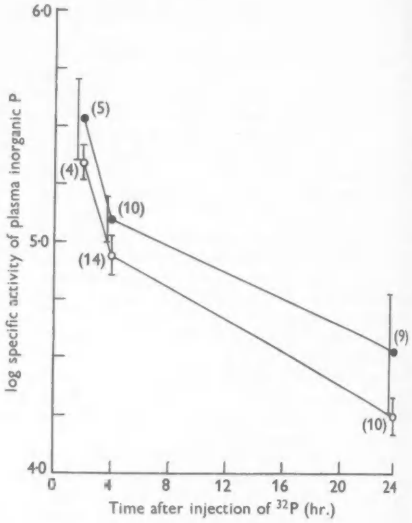

Fig. 2

Fig. 1. Effect of the intraperitoneal injection of 1·2 mg. of triethyltin sulphate/100 g. body wt. at the time indicated by the arrow on the temperature of the liver (○) and brain (●) and on the liver blood flow (△). Liver blood flow is expressed as a percentage of the initial level.

Fig. 2. Disappearance of ^{32}P from the plasma of rats (●) given 1·2 mg. of triethyltin sulphate/100 g. body wt. intraperitoneally 0·75 hr. before the intramuscular injection of 50 μc of ^{32}P/100 g. body wt. and from the plasma of controls (○) given the same dose of ^{32}P and an injection of 0·9 % NaCl equivalent in volume to the dose of triethyltin. The vertical lines show the S.D. of the mean and the numbers in parentheses show the number of rats examined at each point. The means at 4 and 24 hr. after the injection of ^{32}P were significantly different at a level of $P < 0·001$ and $P < 0·01$ respectively.

Table 1. *Effect of triethyltin on the plasma inorganic phosphate level in normal and nephrectomized rats*

Triethyltin sulphate (1·2 mg./100 g. body wt.) was injected intraperitoneally. Bilateral nephrectomy was performed 24 hr. before the experiment.

Treatment	No. of rats	Plasma inorganic P (mg. %)
Normal rats		
Controls	19	8·40 ± 2·27
2 hr. after triethyltin	5	10·43 ± 2·12
4 hr. after triethyltin	10	10·54 ± 1·62*
24 hr. after triethyltin	4	11·96 ± 1·94†
Nephrectomized rats		
Controls	13	25·11 ± 2·47
4 hr. after triethyltin	6	26·30 ± 2·07

* Difference from control mean significant at $P < 0·02$.
† Difference from control mean significant at $P < 0·01$.

control and treated rats was not seen. This suggests that the rise in the plasma inorganic P level after triethyltin in normal rats is due to the associated fall in urine production.

Disappearance of ^{32}P from the plasma

The rate of disappearance of ^{32}P from the plasma after its intramuscular injection was reduced in rats given triethyltin (Fig. 2). Nephrectomized rats were used to see if this was due to a fall in urinary excretion or reduced ^{32}P uptake by the tissues. In six such rats the specific activity of the plasma inorganic P 4 hr. after the intramuscular injection of 25 μc of ^{32}P/100 g. body wt. and 4·75 hr. after the standard dose of triethyltin was 151 900 ± 30 360 counts/min./mg. of P. This was higher ($P < 0·01$) than the corresponding level in the plasma of the 13 nephrectomized controls: 109 800 ± 28 200 counts/min./mg. of P. The condition of these rats was very much worse than that of normal rats given the standard amount of triethyltin. The experiment was therefore repeated in seven rats with a lower dose of triethyltin sulphate (0·3 mg./100 g. body wt.), which produced a similar condition to that in normal rats after the standard dose. Under these conditions the

specific activity of the plasma inorganic P (148 300 ± 44 080 counts/min./mg. of P) was still greater ($P < 0.05$) than in the controls.

Penetration of tissue by ^{32}P after administration of triethyltin

Because of the nature of the lesion produced by triethyltin its effect on the penetration of ^{32}P into the tissues, particularly the brain, is of some interest.

Where the brain was not frozen *in situ*, data on the specific activity of the total acid-soluble P in the brain and spinal cord and on the plasma inorganic P are available from the same animal. In these experiments the ratio of the specific activity of the total acid-soluble P to that of the plasma inorganic P suggested that entry of ^{32}P was hindered by triethyltin, but the differences were not statistically significant (Table 8).

Since cardiac puncture in the anaesthetized rat immediately before immersion in liquid N_2 altered the P levels in the brain the specific activity of the plasma inorganic P could not be compared with the activity of the brain fractions in the same animal. However, if it be assumed that the specific activity of the plasma inorganic P in these rats was the same as in the previous group, the ratios of the specific activity of the total acid-soluble P of the cerebrum in the triethyltin-treated rats and their controls can be compared with the corresponding ratios for the plasma inorganic P (Table 2). If the passage of ^{32}P into the brain was unaltered by triethyltin these ratios would be the same. The results indicate that although ^{32}P penetration during the early stages of triethyltin poisoning was in accordance with the plasma level, it was reduced over a 24 hr. period. The reduced entry of ^{32}P was specially marked when it was examined between 20 and 24 hr. after the injection of triethyltin, i.e. when the rat was severely affected.

The specific activity of the cerebral inorganic P in nephrectomized rats given 0·3 mg. of triethyltin sulphate/100 g. body wt. was compared with that in nephrectomized controls (Table 3) as the inorganic P levels in the plasma and cerebrum of these two groups were the same. The specific activity of the inorganic P of the plasma of the treated rats was 35 % higher than in the controls (see above), but its entry into the total acid-soluble and inorganic P fractions of the cerebrum during a 4 hr. period was reduced by 28 %.

The entry of ^{32}P into the liver appeared unaffected by triethyltin, judging by the specific activity of its total acid-soluble P relative to that of the plasma inorganic P (Table 10).

Effect of triethyltin on distribution of tissue phosphate

There was little change in the three main P fractions of the brain and spinal cord until 24 hr. after the injection of triethyltin, when most of the levels, expressed on a wet-weight basis, had decreased (Table 4). By this time the water content

Table 2. *Ratio of the specific activity of the total acid-soluble phosphate fraction of the cerebrum in rats treated with triethyltin to that in controls*

Triethyltin sulphate (1·2 mg./100 g. body wt.) was injected intraperitoneally. Controls were given an equivalent volume of 0·9 % NaCl; 50 μc of ^{32}P/100 g. body wt. was injected intraperitoneally. The corresponding ratios for the specific activity of the inorganic P of the plasma in a similar group of experimental and control rats are also shown. The number of experimental and control rats in each group is shown in parentheses.

Time after injection (hr.)		Specific activity ratios (triethyltin/control)	
Triethyltin or 0·9 % NaCl	^{32}P	Cerebrum total acid-soluble P	Plasma inorganic P
4·75	4·0	1·53 (3/6)	1·42 (10/14)
24·75	24·0	1·11 (7/6)	2·21 (9/10)
24·0	4·0	0·85 (6/6)	2·76 (8/8)

Table 3. *Effect of triethyltin on the radioactivity of the total acid-soluble and inorganic phosphate fractions of the cerebrum in nephrectomized rats*

All rats were given 25 μc of ^{32}P/100 g. body wt. intramuscularly 4 hr. before they were killed. Rats treated with triethyltin sulphate were given 0·3 mg./100 g. body wt. intraperitoneally and the controls an equivalent volume of 0·9 % NaCl 4·75 hr. before they were killed.

Treatment	No. of rats	Cerebrum (mg. of P/g. wet wt.)		Specific activity (S.A.) (counts/min./mg. of P)		
		Total acid-soluble P	Inorganic P	Total acid-soluble P	Inorganic P	$\frac{10^3 \times \text{S.A. of inorganic P}}{\text{S.A. of total acid-soluble P}}$
Controls	5	0·83 ± 0·25	0·29 ± 0·12	7166 ± 1410	8568 ± 1500	1206 ± 150
Triethyltin	5	0·96 ± 0·62	0·36 ± 0·40	5162 ± 830*	6148 ± 980†	1198 ± 132
Ratio triethyltin/control				0·72	0·72	0·99

* Significantly different from control mean at $P < 0.05$.
† Significantly different from control mean at $P < 0.02$.

Table 4. *Effect of triethyltin sulphate (1·2 mg./100 g. body wt. intraperitoneally) on the distribution of phosphate in the brain and spinal cord of the rat*

The meaning of the figures in parentheses is given in the text.

		Brain				Spinal cord			
			Total acid-soluble P	Lipid P	Residual P		Total acid-soluble P	Lipid P	Residual P
Time after injection of triethyltin (hr.)	No. of rats	Water (%)	(mg. of P/g. wet wt.)			Water (%)	(mg. of P/g. wet wt.)		
Controls	11	78·4±0·08	0·79±0·04	1·77±0·04	0·55±0·06	71·4±0·22	0·75±0·08	2·75±0·24	0·59±0·04
4·75	6	—	0·79±0·04	1·72±0·05*	0·55±0·02	—	0·74±0·17	2·62±0·16	0·61±0·03
24·75	5	80·3±0·21‡	0·71±0·02† (0·73)	1·62±0·08‡ (1·64)	0·52±0·05 (0·51)	76·2±0·59‡	0·70±0·07 (0·64)	2·30±0·08† (2·34)	0·52±0·05* (0·50)

* Significantly different from control mean at $P<0·05$.
† Significantly different from control mean at $P<0·01$.
‡ Significantly different from control mean at $P<0·001$.

Table 5. *Components of the acid-soluble phosphate fraction of the cerebrum of control rats and rats injected with 1·2 mg. of triethyltin sulphate/100 g. body wt. intraperitoneally*

Numbers of rats used are shown in parentheses. PC, phosphocreatine. All results are expressed as mg. of total P/g. wet wt.

Time after injection of triethyltin (hr.)	Total acid-soluble P	Inorganic P	PC	ATP	ADP	AMP	DPN
Controls	0·76±0·16 (9)	0·24±0·09 (13)	0·06±0·007 (13)	0·14±0·02 (15)	0·05±0·008 (16)	0·007±0·004 (16)	0·029±0·005 (16)
4·75	0·65±0·14 (3)	0·10±0·04 (5)	0·09±0·03 (5)	0·15±0·01 (4)	0·05±0·003 (4)	0·006±0·003 (3)	0·032±0·005 (4)
24·75	0·71±0·13 (12)	0·20±0·12 (19)	0·07±0·02 (18)	0·14±0·03 (20)	0·05±0·011 (20)	0·006±0·003 (19)	0·025±0·007 (19)
24·75 (corrected for oedema)	0·78	0·22	0·08	0·15	0·05	0·007	0·027

of the brain and spinal cord had increased significantly, owing to the addition of an ultrafiltrate of plasma to these tissues without change in their solid elements (Magee *et al.* 1957). Values (shown in parenthesis in Table 4) for the P fractions 24 hr. after the administration of triethyltin can therefore be calculated from the control levels, as described in the Appendix of Magee *et al.* (1957). Their agreement with the experimental values suggests that the observed changes were due to the oedema.

In the further fractionation of the acid-soluble P fraction of the cerebrum (Table 5) the variability of the inorganic P fraction could not be attributed to changes in labile P compounds. Although the samples were freed from bone as carefully as possible this variation is thought to be due to the presence of undetected spicules which had contaminated the cerebrum during its dissection from the frozen head. Triethyltin had no significant effect on the levels of the other components of this fraction even when allowance was made for the cerebral oedema. There was no loss of phosphocreatine or ATP even in rats killed on the point of death.

The water content of the liver was unaffected by triethyltin and 4 hr. after its administration the wet weight of the liver/100 g. body wt. was the same as in the controls. The blood content of the liver in the rats treated with triethyltin did not differ from that in the controls and was similar to that found by others with different methods (Caster, Simon & Armstrong, 1955). The lipid and residual P fractions were unaltered during the first few hours after the injection of triethyltin, but the acid-soluble P level rose significantly (Table 6). The latter is seen also in another group of rats in Table 7. There, although the rise in the acid-soluble P fraction was similar, the control level was much lower. The difference in the control levels could not be explained on technical grounds and may be due to differences in the animals due either to the change in diet or the different season of the year in which they were obtained. The increase in the total acid-soluble P level after triethyltin could not be accounted for by the rise in the plasma inorganic P but corresponded with the increases in the ATP and ADP levels, which reached a maximum about this time (Table 7). Further evidence for an increase in the liver nucleotide content 4 hr. after the injection of triethyltin was obtained by examining the u.v.-light-absorption spectra of the liver extracts. The extinction (1 g./ml.) at 260 mμ was $125 \pm 7{\cdot}4$ for six control extracts and $151 \pm 7{\cdot}4$ for extracts from six treated rats. This difference is highly significant ($P < 0{\cdot}001$) and, with no chromatographic evidence of a decrease in the non-adenine nucleotides, suggests an increase in the nucleotide content of the liver. With a value of $1{\cdot}47 \times 10^4$ for the molar-absorbency index (Bock, Ling, Morell & Lipton, 1956), the increase in extinction at 258 mμ represents an increase of 238 μg./g. in the adenine content of the liver, more than sufficient to account for the observed increase in adenine nucleotide and total P levels. The progressive nature of the change in the adenine nucleotide of the acid-soluble fraction of the liver is shown in Table 7. This change did not extend to the DPN or TPN fractions but was reflected in the increase in the uridine triphosphate and guanosine triphosphate levels.

Histological examination of the liver revealed a wave of mitoses occurring about 4 hr. after the injection of triethyltin. This was the only abnormal feature seen in these acute experiments otherwise the appearance of the liver was normal, as after much longer periods of exposure to triethyltin (Magee *et al.* 1957).

Effect of triethyltin on distribution of ^{32}P *in the nervous system*

To circumvent difficulties arising from the unequal penetration of ^{32}P into the nervous systems of control and experimental rats the specific activities of the P fractions within the tissue have been expressed as fractions of the specific activity of the total acid-soluble P as done by Webster (1954). The pattern of incorporation of ^{32}P in the spinal cord of these young rats differed from that found by Webster (1954) in adult hens. This was not due to the slight differences in analytical technique, since when this technique was applied to adult hens the results agreed with those of Webster.

Table 6. *Effect of triethyltin sulphate (1·2 mg./100 g. body wt. injected intraperitoneally) on distribution of phosphate in the liver of the rat*

Time after injection of triethyltin (hr.)	No. of rats	Total acid-soluble P	Lipid P	Residual P
		(mg. of P/g. wet wt.)		
Controls	10	$1{\cdot}19 \pm 0{\cdot}06$	$1{\cdot}25 \pm 0{\cdot}09$	$1{\cdot}29 \pm 0{\cdot}08$
2·75	5	$1{\cdot}30 \pm 0{\cdot}07$*	$1{\cdot}26 \pm 0{\cdot}12$	$1{\cdot}31 \pm 0{\cdot}08$
4·75	7	$1{\cdot}28 \pm 0{\cdot}04$*	$1{\cdot}29 \pm 0{\cdot}04$	$1{\cdot}34 \pm 0{\cdot}05$

* Significantly different from control mean at $P < 0{\cdot}01$.

Table 7. *Components of the acid-soluble phosphate fraction of the liver of control rats and of rats after intraperitoneal injection of 1·2 mg. of triethyltin sulphate/100 g. body wt.*

Numbers of rats used are shown in parentheses. GTP, guanosine triphosphate; UTP, uridine triphosphate. All results are expressed as mg. of total P/g. wet wt.

Group	Time after injection of triethyltin (hr.)	Total acid-soluble P	ATP	ADP	AMP	DPN	TPN	GTP	UTP
Controls	—	0·96±0·05 (17)	0·144±0·026 (22)	0·081±0·010 (22)	0·016±0·006 (22)	0·044±0·006 (21)	0·020±0·005 (22)	0·016±0·003 (6)	0·011±0·002 (6)
Triethyltin	1	0·99 (2)	0·197±0·017* (3)	0·083±0·006 (3)	0·011±0·002 (3)	0·043±0·005 (3)	0·016±0·003 (3)	—	—
Triethyltin	2	1·02±0·11 (3)	0·198±0·020† (5)	0·088±0·006 (5)	0·012±0·006 (5)	0·042±0·003 (5)	0·016±0·003 (5)	—	—
Triethyltin	4	1·12±0·05† (18)	0·226±0·026† (22)	0·090±0·011* (22)	0·014±0·004 (22)	0·047±0·005 (22)	0·021±0·004 (22)	0·020±0·003‡ (6)	0·016±0·003* (6)
Triethyltin	24	1·12±0·04† (4)	0·254±0·047† (4)	0·106±0·014† (4)	0·015±0·002 (4)	0·045±0·010 (4)	0·023±0·005 (5)	—	—

* Significantly different from control mean at $P<0·01$.
† Significantly different from control mean at $P<0·001$.
‡ Significantly different from control mean at $P<0·05$.

Table 8. *Effect of triethyltin on the radioactivity of the phosphate fractions of the brain and spinal cord*

Triethyltin sulphate (1·2 mg./100 g. body wt.) was injected intraperitoneally; 50 μc of ^{32}P/100 g. body wt. was injected intramuscularly. Results are expressed as the relative specific activity, which for the total acid-soluble P = [(counts/min./mg. of total acid-soluble P)/(counts/min./mg. of plasma inorganic P)] × 1000 and for the lipid and residual P = [(counts/min./mg. of lipid or residual P)/(counts/min./mg. of total acid-soluble P)] × 1000.

Time after injection (hr.)		No. of rats	Relative specific activity					
			Brain			Spinal cord		
Triethyltin	^{32}P		Total acid-soluble P	Lipid P	Residual P	Total acid-soluble P	Lipid P	Residual P
Control	4·0	6	82·3±14·9	49·7±8·1	150·3±23·4	106·7±19·4	36·0±4·8	173·4±34·1
Control	24·0	5	414·4±81·2	185·8±13·0	261·8±29·4	506·3±122·2	127·7±9·3	318·0±21·5
4·75	4·0	6	66·3±15·3	32·3±7·9*	139·0±25·0	87·5±24·2	22·3±6·5*	129·3±18·7†
24·75	24·0	5	280·3±154·6	89·2±18·3‡	195·3±14·7*	342·4±209·6	61·0±12·4‡	211·1±22·2‡

* Significantly different from control mean at $P<0·01$.
† Significantly different from control mean at $P<0·05$.
‡ Significantly different from control mean at $P<0·001$.

Triethyltin depressed the relative specific activities of the lipid and residual P fractions in both brain and spinal cord, the changes being most marked in the latter (Table 8). Although the relative specific activity of the total acid-soluble P was lower in the rats given triethyltin its variability was such that the differences were not statistically significant. Within this fraction distribution of ^{32}P in the energy-rich phosphates (phosphocreatine, ATP and ADP) of the cerebrum was unaffected by triethyltin even when ^{32}P was not given until 20 hr. after the injection of triethyltin, i.e. when the rat was extremely ill (Table 9). The incorporation of ^{32}P into the guanosine and uridine triphosphates of the cerebrum was also unaffected by triethyltin in the few experiments in which they were measured. The only striking change was the reduction in the relative activity of the DPN. The concentrations of DPN and adenosine monophosphate in the cerebrum are very low and their rate of labelling is slow so that reliable data could only be obtained 24 hr. after the injection of ^{32}P.

Effect of triethyltin on distribution of ^{32}P in the liver

The distribution of ^{32}P within the liver has been expressed in terms of relative specific activity as for the brain. The relative specific activities of the total acid-soluble P and its constituents were unaltered by triethyltin (Tables 10 and 11). As in

Table 9. *Effect of triethyltin on the relative specific activity of the components of the acid-soluble phosphate fraction of the cerebrum of the rat*

Triethyltin sulphate (1·2 mg./100 g. body wt.) was injected intraperitoneally; 50 μc of ^{32}P/100 g. body wt. was injected intramuscularly. Relative specific activity = (specific activity of total P of compound/specific activity of total acid-soluble P) × 1000. PC, phosphocreatine.

Time after injection (hr.)		No. of rats	Relative specific activity					
Triethyltin	^{32}P		Inorganic P	PC	ATP	ADP	AMP	DPN
Control	4·0	6	1480±330	1140±610	910±350	780±110	—	—
Control	24·0	6	1100±160	1120±390	1020±140	920±160	1190±490	680±70
4·75	4·0	3	2010±310	1230±200	910±90	630±100	—	—
24·0	4·0	6	1960±570	900±220	828±170	600±170	—	—
24·75	24·0	7	1334±180*	1290±270	980±170	880±150	920±230	360±220†

* Significantly different from control mean at $P < 0.05$.
† Significantly different from control mean at $P < 0.01$.

Table 10. *Effect of triethyltin on the radioactivity of the phosphate fractions of the liver*

Triethyltin sulphate (1·2 mg./100 g. body wt.) was injected intraperitoneally; 50 μc of ^{32}P/100 g. body wt. was injected intramuscularly. Results are expressed as the relative specific activity, which for the total acid-soluble P = [(counts/min./mg. of total acid-soluble P)/(counts/min./mg. of plasma inorganic P)] × 1000 and for the lipid and residual P = [(counts/min./mg. of lipid or residual P)/(counts/min./mg. of total acid-soluble P)] × 1000.

Time after injection (hr.)		No. of rats	Relative specific activity		
Triethyltin	^{32}P		Total acid-soluble P	Lipid P	Residual P
Controls	2	4	861·8±197·3	138·7±31·7	40·8±6·7
Controls	4	6	1279·2±220·5	368·1±59·9	75·5±5·6
2·75	2	4	757·4±371·0	101·2±31·7	45·4±8·5
4·75	4	7	1311·6±292·8	257·1±34·4*	87·1±7·0†

* Significantly different from control mean at $P < 0.001$.
† Significantly different from control mean at $P < 0.01$.

Table 11. *Effect of triethyltin on the relative specific activity of the nucleotides in the liver of the rat*

Rats were killed 1 hr. after 25 μc of ^{32}P/100 g. body wt. had been injected intramuscularly and 4 hr. after the intraperitoneal injection of either 1·2 mg. of triethyltin sulphate/100 g. body wt. or an equivalent volume of 0·9% NaCl.

Group	No. of rats	Relative specific activity				
		ATP	ADP	AMP	DPN	TPN
Control	6	1504·2±576·5	1016·5±100·3	713·3±129·2	95·1±18·4	332·7±120·6
Triethyltin	6	1326·1±204·9	1153·9±150·9	595·0±117·1	114·8±30·8	291·2± 84·5

* Significantly different from control mean at $P < 0.05$.
† Significantly different from control mean at $P < 0.01$.
‡ Significantly different from control mean at $P < 0.001$.

the nervous system the incorporation of ^{32}P into the lipid P fraction was reduced but there was an increase in the relative activity of the residual P fraction (Table 10).

DISCUSSION

The striking pathological finding in triethyltin poisoning is the interstitial oedema of the central nervous system without visible damage to the nerve cells or demyelination of nerve tracts. Therefore it was perhaps rather surprising to find the entry of ^{32}P into the nervous system impeded by triethyltin, although this confirms previous results on the penetration of sulphanilamide after triethyltin (Magee *et al.* 1957). As this defect is to some extent correlated with the general condition of the rat it might be related to a reduced circulation of blood through the tissues with a poor distribution of ^{32}P to them. However, its entry into the liver was unaffected, and this is unlikely to be the whole explanation for if any significant cerebral ischaemia existed the phosphocreatine and ATP levels would have fallen, and such falls were never found. Furthermore, there was no evidence that triethyltin affected hepatic blood flow during the period under discussion and the flow through the brain is usually better preserved than through the liver (Stoner, 1954). The difference may be due to a failure in a transport mechanism whereby the ^{32}P crosses some 'membrane' into the central nervous system. Further work is planned on these problems and it may be wrong to seek quite such a specific explanation. After triethyltin the temperature falls in both the brain and the liver and the reduced penetration of ^{32}P into the brain could be due to the blood–brain barrier being more sensitive to temperature change than the blood–liver barrier.

Once the ^{32}P has entered the brain its distribution to the energy-rich phosphates, phosphocreatine, ATP and ADP, appears unimpaired. From previous work with ^{32}P (Stoner, 1950; Stoner *et al.* 1952) it seems likely that in a tissue in a living animal where oxidative phosphorylation is occurring the incorporation of ^{32}P into these compounds is so rapid that partial inhibition of the process would not be seen in such results. This is particularly true for the brain because of the slow rate of entry of the isotope (Streicher, 1956). 2:4-Dinitrophenol strongly inhibits oxidative phosphorylation (Loomis & Lipmann, 1948) *in vitro*, but fatal doses of the related 3:5-dinitro-*o*-cresol have no effect on the specific activity of ^{32}P in the energy-rich phosphates of rat skeletal muscle *in vivo* (Stoner *et al.* 1952). Nevertheless, the tissue levels of these phosphates are reduced in rats poisoned with 3:5-dinitro-*o*-cresol, particularly in the brain (Parker, 1954), showing that it does interfere with their metabolism. Rats given triethyltin showed no change in the amount of phosphate in the various fractions of the central nervous system, and the findings in the present experiments would not have suggested that triethyltin inhibited oxidative phosphorylation *in vitro*. In individual experiments the phosphocreatine and ATP levels in the cerebrum were often higher than in the controls, suggesting that the reduced activity of the rat after triethyltin had a protective effect on the levels of these compounds, as with the narcotics discussed by Buchel & McIlwain (1950).

The observed effects of triethyltin on the incorporation of ^{32}P into the nervous system, namely the reduction in the relative specific activities of the DPN, lipid P and residual P fractions, suggest interference with the utilization of chemical energy. Although ^{32}P can be incorporated into the energy-rich phosphates its further distribution is impaired. In this the action of triethyltin resembles that of sodium pentobarbital (Dawson & Richter, 1950) and chlorpromazine (Ansell & Dohmen, 1956). This similarity may be merely a reflexion of the fall in tissue temperature produced by all those substances.

If all the changes discussed so far are secondary to the fall in tissue temperature attention is focussed on the mechanism of that hypothermia. With triethyltin this will be complex. As it is a peripheral vasodilator (Stoner *et al.* 1955) heat loss will be accelerated and not all of the effect will be due to decreased heat production. It should also be recalled that when heat loss is reduced by raising the environmental temperature to 30° the symptoms of triethyltin poisoning in the rat are exaggerated (Stoner *et al.* 1955). The way in which energy is liberated as heat during oxidation in the tissues is incompletely understood, as is the way in which this process is controlled. Metabolic inhibitors fall into two groups, according to whether they lower or raise body temperature, and their effect on tissue nucleotides may well be determined by this. With triethyltin, chlorpromazine and the barbiturates the fall in tissue temperature, by lowering the demand for metabolic energy, may allow compensatory mechanisms to preserve the levels of phosphocreatine and ATP. For 3:5-dinitro-*o*-cresol this compensation cannot be effective because of the rise in tissue temperature (Stoner, 1956).

The interpretation of the action of triethyltin in the whole animal is further complicated by the changes in the liver, where the concentration of triethyltin is much higher than in the brain (Cremer, 1957). The incorporation of ^{32}P into the lipid P was again impaired but the amount of acid-soluble adenine nucleotide in the liver was increased, as was the relative specific activity of the residual P fraction. Further studies on these last

two changes are in progress. The difficulty in interpreting changes in the radioactivity of the residual P is discussed by Davidson *et al.* (1951), particularly in regard to its possible contamination from other fractions. In so far as these results indicate a difference in the responses of the liver and brain they are in agreement with those of Cremer (1957), yet the isolated mitochondria from these tissues are equally sensitive to triethyltin (W. N. Aldridge, personal communication). It would be premature to speculate on the mechanism of these changes which occur in the liver while its temperature is falling.

At the moment these experiments give little help towards understanding the mechanism of action of triethyltin in the whole animal. They do, however, illustrate some of the difficulties which may arise in trying to explain the toxicity of metabolic inhibitors and in trying to correlate studies *in vitro* with studies *in vivo*.

SUMMARY

1. The effect of fatal doses of triethyltin sulphate on the levels of the tissue phosphates and the distribution of injected ^{32}P has been studied in the rat.

2. The plasma inorganic phosphate level rose after triethyltin and this was attributed to a lowered renal function. The specific activity of the plasma inorganic phosphate was higher in the rats treated with triethyltin than in the controls and the penetration of the ^{32}P into the brain was reduced. Its penetration into the liver was unaltered.

3. The phosphate levels in the central nervous system were unaffected by triethyltin except in so far as they were altered by the changes in water content due to the accumulating interstitial oedema. The adenosine triphosphate and diphosphate levels in the liver increased within a few hours of injecting triethyltin.

4. In the central nervous system the relative specific activities of diphosphopyridine nucleotide, the lipid phosphate and the residual phosphate were reduced after triethyltin. In the liver the relative specific activity of the lipid phosphate was also reduced but that of the residual phosphate fraction was increased.

5. These findings are discussed in their relation to the pharmacological effects of triethyltin and the results of previous work in this Laboratory on the action of triethyltin on isolated systems. No conclusion could be reached concerning the mechanism of action of triethyltin in the whole animal but it is thought that the hypothermic action of triethyltin may play an important part in producing the changes observed in the brain.

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The Activation and Inhibition of 5-Nucleotidase

By Z. AHMED and J. L. REIS
Department of Chemical Pathology, Postgraduate Medical School, London, W. 12

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The activation and inhibition of 5-nucleotidase have not been much studied. According to previous papers by Reis (1937, 1950) magnesium activates this enzyme to a much smaller extent than it does the non-specific phosphomonoesterase 'alkaline phosphatase'. Kaye (1955) mentions that zinc inhibits 5-nucleotidase, but his evidence is scanty.

We have limited our study to human tissues, and have used aorta wall as a source of 5-nucleotidase, where it is free from non-specific phosphatase activity, and placenta, which has both enzymes.

EXPERIMENTAL

pH. The enzyme-activity measurements were carried out at pH 7·5 (Reis, 1951). This pH is near the optimum for 5-nucleotidase (pH 7·8), whereas the non-specific phosphatases display very low activity at this hydrogen-ion concentration.

Substrates. By using phenyl phosphate as substrate we obtained values for the non-specific phosphatase activity. With 'muscle' adenylic acid (adenosine 5′-monophosphate) the values represent the sum of 5-nucleotidase and non-specific phosphatase activities. By subtraction of the latter the probable 5-nucleotidase activity is obtained.

Enzyme solutions. Human aorta was taken at autopsy about 24 hr. after death. The extracts were prepared from the media after removal of the intima and adventitia. Human placenta was obtained within 2 hr. of its delivery, and cleaned and perfused with tap water. The tissues were homogenized in a blender with 10 vol. of water and left for 3 days at room temperature with a few drops of chloroform. After centrifuging, the supernatant was used as tissue extract.

Estimations. Centrifuge tubes containing 1·4 ml. of 0·05 M-veronal buffer of pH 7·5, 0·2 ml. of 0·01 M-substrate (sodium phenyl phosphate or adenylic acid, adjusted to pH 7·5) and 0·2 ml. of activator or inhibitor solution (pH 7·5) were placed in a water bath for about 5 min. at 38°. Then 0·2 ml. of enzyme solution was added (tissue extract) and the sample incubated. To stop enzyme activity 4 ml. of 5% (w/v) trichloroacetic acid was added. After 5 min. the sample was centrifuged and the free phosphate estimated by the method of Fiske & Subbarow (1925).

Table 1. *Activation and inhibition of aortic 5-nucleotidase*

The results are represented as μg. of P liberated. They correspond to the activity of 20 mg. of tissue during 20 min., at pH 7·5 and 38°. The phenyl phosphate hydrolysis under these conditions was only 0·2 μg. of P, so it was ignored and the adenylic acid hydrolysis regarded as due only to 5-nucleotidase activity.

	Ions added (final concn. mM)							
	None	Mg^{2+}	Mn^{2+}	Zn^{2+}	Co^{2+}	Ni^{2+}	PO_4^{3-}	Adenosine
Adenylic acid hydrolysis (5-nucleotidase activity)	9·1	10·2	14·5	1·0	2·2	0·3	8·6	8·9

Table 2. *Activation and inhibition of placental phosphatases*

The results are represented as μg. of P liberated. They correspond to the activity of 10 mg. of tissue during 20 min., at pH 7·5 and 38°.

	Ions added (final concn. mM)											
	None	Mg^{2+}	Mn^{2+}	Ca^{2+}	Ba^{2+}	Co^{2+}	Cu^{2+}	Zn^{2+}	Ni^{2+}	CN^-	F^-	PO_4^{3-}
Phenyl phosphate hydrolysis (non-specific phosphatase activity)	9·0	9·7	10·2	9·7	10·2	10·2	9·2	9·0	9·0	9·0	10·0	4·8
Adenylic acid hydrolysis (non-specific phosphatase and 5-nucleotidase activity)	22·3	22·8	27·1	22·0	25·6	14·6	14·6	9·4	9·0	22·8	23·5	18·9
Hydrolysis due to 5-nucleotidase activity (calc. by subtraction)	13·3	13·3	16·9	12·3	15·4	4·4	5·4	0·4	0·0	13·8	13·5	14·1

RESULTS

In Tables 1 and 2 the results obtained with different ions are shown. The influence of different ions on the 5-nucleotidase from aorta and from placenta (where it occurs together with phosphatase) is the same, which points to the identity of aortic and placental 5-nucleotidase. Magnesium, the well-known activator of phosphatases, has only a slight action on 5-nucleotidase. The highest activation of 5-nucleotidase was caused by manganese (60 %).

A very strong inhibition was obtained with Zn^{2+} and Ni^{2+} ions. Zince sulphate precipitated the enzyme from solution, so it could not be excluded that this precipitation was the cause of inhibition. Nickel did not cause any precipitation and inhibited 5-nucleotidase sometimes to a greater extent than zinc did. A millimolar solution of nickel salts inhibited 5-nucleotidase almost totally, while not affecting at all non-specific phosphatase activity (Figs. 1, 2).

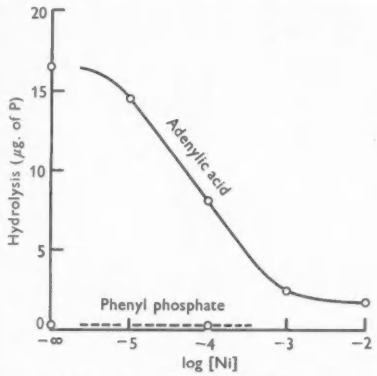

Fig. 1. Inhibition of aortic 5-nucleotidase by nickel. (20 mg. of tissue, 30 min., pH 7·5, 38°.)

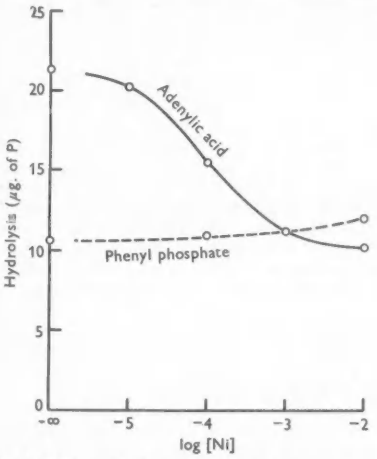

Fig. 2. Inhibition of placental 5-nucleotidase by nickel. (10 mg. of tissue, 20 min., pH 7·5, 38°.)

This inhibition by Ni^{2+} ions could be used for estimations of 5-nucleotidase activity. The present method consists in subtracting from the rate of hydrolysis of adenylic acid the rate of hydrolysis of phenyl phosphate at pH 7·5, on the assumption that adenylic acid is hydrolysed by 5-nucleotidase and non-specific phosphatase, whereas phenyl phosphate is hydrolysed by non-specific phosphatase only. Since the alkaline phosphatase hydrolyses phenyl phosphate more quickly than it hydrolyses many other phosphoric esters (King & Delory, 1939), including adenylic acid, an error is introduced by subtracting too high values, thus obtaining too low values for 5-nucleotidase activity. By using β-glycerophosphate for the same purpose an error is made in the opposite direction, as this ester is hydrolysed by alkaline phosphatase more slowly than is adenylic acid. What we should subtract is the rate of hydrolysis of adenylic acid due only to the non-specific phosphatase. A more direct estimation of 5-nucleotidase can be obtained by using inhibition of 5-nucleotidase by nickel.

Inhibition by phosphate has also been tried, as phosphate is a competitive inhibitor of phosphatases. Our results show that 5-nucleotidase is much less inhibited by phosphate than is the alkaline phosphatase. Adenosine in concentration equal to that of the substrate had no inhibitory action.

SUMMARY

1. 5-Nucleotidase is activated strongly by Mn^{2+} ions.

2. Nickel (Ni^{2+}) and Zn^{2+} ions inhibit 5-nucleotidase activity. Ni^{2+} in millimolar concentration almost completely inhibits the 5-nucleotidase, whereas non-specific phosphatase activity is not affected. The possibility of using nickel inhibition for 5-nucleotidase estimations is discussed.

3. Phosphate inhibits 5-nucleotidase to a much smaller extent than it does alkaline phosphatase.

The authors wish to thank Professor E. J. King for his advice.

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The Mechanism of Polysaccharide Production from Sucrose

5. TRANSFER OF FRUCTOSE TO C-1 OF ALDOSE BY LEVANSUCRASE*

By S. HESTRIN and G. AVIGAD

Laboratory of Microbiological Chemistry, Department of Biochemistry, Hebrew University—Hadassah Medical School, Jerusalem, Israel

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Levansucrase catalyses the reversible transfer of fructose from an aldosyl β-fructofuranoside (R~fr) to C-1 of free aldose (R—H) (eqn. 1):

$$R_1 \sim fr + R—H \rightleftharpoons R \sim fr + R_1—H. \quad (1)$$

This enzymic ability has enabled a series of new sucrose analogues to be synthesized (Hestrin, Feingold & Avigad, 1955; Avigad, Feingold & Hestrin, 1956; Feingold, Avigad & Hestrin, 1957; Avigad, 1957; Péaud-Lenoël & Dedonder, 1957; Péaud-Lenoël, 1957*a*).

This paper defines the range of eqn. 1. A preliminary account of some of these experiments has been presented (Avigad & Hestrin, 1957). An example (eqn. 2) of the aldosyl exchange reaction is now described quantitatively:

$$\text{Sucrose} + \text{melibiose} \rightleftharpoons \text{raffinose} + \text{glucose} \quad (2)$$

Competitive inhibition of levansucrase by methyl α-D-glucopyranoside and methyl β-D-fructofuranoside is also demonstrated. To account for the specificity of levansucrase towards substrates and inhibitors, the idea is advanced that the active surface of this enzyme comprises an aldophilic alongside a fructophilic centre.

Nomenclature. The relationship to sucrose of compounds (R~fr) in which the glucose moiety has been replaced by another aldose is indicated by attaching a prefix to sucrose. Thus α-galactosyl β-fructofuranoside is named 'galsucrose'; α-lactosyl β-fructofuranoside (4^G-β-galactosylsucrose) is named 'lactsucrose', etc.

MATERIALS AND METHODS

Enzyme. Levansucrase was prepared from cells of *Aerobacter levanicum* grown on sucrose medium as described previously (Hestrin, Feingold & Avigad, 1956). The enzyme solution formed 20 mg. of levan/hr./mg. of protein N (0·15 M-sucrose, 37°, pH 5·4).

Aldoses. Many aldoses and several sucrose analogues were provided by the generosity of colleagues (see acknowledgements). Uniformly labelled [^{14}C]sucrose and [^{14}C]-glucose were obtained from the Radiochemical Centre, Amersham, Bucks. Fructosylglucose isomers (Feingold *et al.* 1956) and R~fr compounds which differed from sucrose at C-4, C-5 or C-6 of the aldose moiety were prepared enzymically by the action of levansucrase. D-Xylulose and D-ribulose were tested as a mixture (hydrolysate of the equilibrium mixture of products formed from ribose 5-phosphate by pentose phosphate isomerase). Methyl β-D-fructofuranoside was prepared as described by Breuer & Bacon (1957).

Dextran (Commercial Solvents Corp., Terre Haute, Ind., U.S.A.) and glycogen (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) were hydrolysed partially in acid as described by Barker, Bourne, James, Neely & Stacey (1955), and xylan (Pfanstiehl Chemical Co., Waukegan, Ill., U.S.A.) as described by Whistler & Tu (1952). The partial hydrolysates were partially desalted, concentrated and finally freed of monoses and residual salt by washing with water on a carbon–Celite column. Solutions of the homologous polymer series were obtained by extracting the extruded contents of the columns in 50% (v/v) ethanol and concentrating the extracts under reduced pressure.

The purity of each of the substrates used was checked by means of paper chromatography. Commercial preparations of L-xylose and D-arabinose were shown to be substantially pure substances by measurement of the optical rotation.

Standard conditions. Digests contained 0·1 M-donor (sucrose, raffinose) and approx. 10% (w/v) acceptor in phosphate–citrate buffer [McIlvaine solution, pH 5·4, diluted 1:5; Gomori, 1955) at 37° under toluene. The enzyme concentration was 0·5 unit/ml. (unit as defined by Dedonder & Péaud-Lenoël, 1957), representing 0·02% protein N.

Technique of survey of carbohydrates. Depending on chromatographic convenience, either a disaccharide (sucrose) or a trisaccharide (raffinose) was employed as the donor system. Raffinose was used in tests of hexoses, sucrose in tests of oligoaldoses and either sucrose or raffinose in tests of pentoses, heptoses and deoxy sugars. Acceptor activities of the carbohydrates were surveyed in sets of parallel experiments, each with a control test with either glucose (raffinose as donor) or melibiose (sucrose as donor). Under the conditions selected as standard (see above), the enzyme was at saturation in relation to glucose and at about half saturation in relation to melibiose. To conserve rare carbohydrates, microtechniques were employed. The reaction vessel consisted of glass capillary tubes (1·5 mm. diam.) and contained a total volume of reaction mixture of 30–60 μl. (Hestrin *et al.* 1956). Portions (4–10 μl., containing 0·5–1·0 mg. of total carbohydrate) were removed with the aid of calibrated capillary pipettes (0·5 mm. diam.), applied to Whatman no. 1 filter paper along a start line in spots of approx. 4 mm. diam. and immediately dried in a stream of warm air. Mercuric

* Part 4: Feingold, Avigad & Hestrin (1956).

chloride incorporated previously into the paper (0·05 μmole of mercuric chloride/spot) ensured the prompt arrest of the reaction.

Solvent systems and spray reagents are listed in Table 1. Chromatograms were resolved at room temperature (approx. 25°). The minimum amount of fructose which could be detected on paper by means of the urea–phosphoric acid reagent was found to be 3 μg. (free or combined).

A product is described in this paper as an aldosylfructoside only if it exhibited the following behaviour: ketose test, +; aldose test, +; test for reducing-group activity, −; mobility quotient (mobility of free aldose/mobility of formed aldosylfructoside), 1·57±0·05 (in solvent *b* (Table 1)); the same quotient measured in solvent *a* was 1·42±0·05. Aldosylfructoses and fructosylaldose could be differentiated readily from aldosylfructosides on the basis of the test for reducing-group activity.

Some of the aldoses examined were of types (pentose, deoxyhexose, methylpentose and heptose) which gave differentiating colour reactions with listed spray reagents. With these the results of the spray tests provided a direct confirmation of the assumption that the aldose added to a test system had been incorporated into the formed aldosylfructoside.

Analyses of radioactive substrates. Components of ^{14}C systems were resolved chromatographically as described above. Portions were adjusted to contain 83 μmc±10%, unless otherwise is indicated. The distribution of radioactivity on the paper chromatogram was recorded (Roberts & Carleton, 1956) with a model C100 Actigraph with a D34 thin-window counter (Nuclear Chicago Co., Chicago, Ill., U.S.A.). A slit width of $\frac{1}{4}$ in. was used on the 250 scale and one of $\frac{1}{8}$ in. on the 1K scale range of the rate meter. The recordings were analysed planimetrically. To estimate an area beneath a peak, a base line was drawn at a level corresponding to the background activity. The total area enclosed by the base line and the traced curve was taken as 100, the increments contributed by the single peaks being expressed as percentages. Overlapping areas under successive peaks were estimated by extrapolation. In the systems in which ^{14}C had been supplied in the form of uniformly labelled [^{14}C]sucrose, relative molar concentrations were calculated from radioactivities on the basis of the following relationship (A = counts/min.; M = moles): M (fructose): M (sucrose): M (1^F-β-fructosylsucrose) = A (fructose): $\frac{1}{2}A$ (sucrose): $\frac{1}{3}A$ (1^F-β-fructosylsucrose). The experimental results were in good accord with this theoretically expected relationship (see Hestrin *et al.* 1956). Planimetric measurements of components representing ⩾5% of total ^{14}C were found to be reproducible within ±5%. Components were identified by both their mobility and reactions with spray reagents.

In several of the experiments, paper regions corresponding to radioactivity peaks were eluted with hot water and assayed for fructose colorimetrically (Roe, Epstein & Goldstein, 1949). The colorimetric and planimetric assay were found to agree within ±6%. Internal checks on the validity of planimetric assays were obtained by demonstrating that in systems in which [^{14}C]sucrose was the sole source of added ^{14}C the results of radioactivity measurements conformed within <5% to predicted relationships, namely the following (A = counts/min.): A (Δ sucrose) = A (products formed from sucrose); A (glucose) = $\frac{1}{2}$[A (Δ sucrose) − $\frac{2}{3}A$ (1^F-β-fructosylsucrose)]; A (glucose) = A (fructose) + A (levan) + $\frac{1}{3}A$ (1^F-β-fructosylsucrose).

RESULTS

Survey of aldoses

On the basis of the net amounts of aldosylfructoside found to be formed from aldoses in standard reaction conditions, three classes of aldose were distinguished (numbers in parenthesis give the range of yield of aldosylfructoside in μmoles/ml., as observed at 30–60% decomposition of donor): (*a*) apparently inert compounds (<1·5); (*b*) poor acceptors (1·5–5·0); (*c*) good acceptors (⩾20).

Table 1. *Methods used in paper chromatography*

Solvent systems

Designation	Composition of solvent system (by vol.)	Reference	Flow time (hr.)	Type of carbohydrate resolved
a	Propanol–ethyl acetate–water (7:1:2)	Albon & Gross (1952)	24–36	Neutral
b	Butanol–ethanol–water (5:2:2)	—	60–70	Neutral
c	Pyridine–ethyl acetate–acetic acid–water (5:5:1:3)	Fischer & Dörfel (1955)	12	Uronic acid, lactone
d	Propanol–aq. NH_3 soln.–water (6:3:1)	Hanes & Isherwood (1949)	10	Phosphate ester*

* Papers were pretreated with ethylenediaminetetra-acetic acid (Eggleston & Hems, 1952).

Spray tests

Component located	Spray reagent	Reference
Neutral sugar		
Reducing group	Triphenyltetrazolium chloride (alkaline)	Wallenfels (1950)
	Aniline oxalate	Horrocks & Manning (1949)
Ketose	Urea–phosphoric acid	Wise, Dimler, Davis & Rist (1955)
Aldose	Aniline oxalate	Horrocks & Manning (1949)
Uronic acid	*p*-Anisidine	Hough, Jones & Wadman (1950)
Phosphate ester	Ammonium molybdate	Hanes & Isherwood (1949)

Table 2. *Compounds tested for acceptor activity at* C-1 *in respect of fructose transferred from sucrose or raffinose*

Definitions of classes (*a*), (*b*) and (*c*) are given in the text. The figure in parenthesis after the name of an aldose is the $R_{sucrose}$ (solvent *b*) of the aldosylfructoside formed.

Compound*	Class *a*	Class *b*	Class *c*
Aldopentose	D-Ribose	D-Arabinose (1·12)	D-Xylose (1·20)
	D-Lyxose	L-Xylose (1·18)	L-Arabinose (1·14)†
Aldohexose	D-Mannose	L-Glucose (1·05)	D-Glucose (1·00)
	D-Gulose	L-Galactose (0·90)	D-Galactose (0·92)
	D-Talose	D-Altrose (1·26)	—
	—	D-Idose (1·32)	—
Aldoheptose	D-Glycero-L-mannose	D-Glycero-L-glucose (0·66)	D-Glycero-D-galactose (0·60)
	D-Glycero-D-gulose	D-Glycero-L-galactose (0·57)	—
Aldo-octose	—	D-Threo-L-galactose (0·58)	—
Ketopentose	D-Ribulose	—	—
	D-Xylulose	—	—
Ketohexose	D-Fructose	—	—
	L-Sorbose‡	—	—
	D-Tagatose	—	—
Ketoheptose	D-Mannoheptulose	—	—
	D-Glucoheptulose	—	—
Deoxyaldose	2-Deoxy-D-ribose	—	—
	2-Deoxy-D-glucose	—	—
	2-Deoxy-D-allose	—	—
	6-Deoxy-L-mannose (L-rhamnose)	6-Deoxy-L-galactose (L-fucose) (1·52)	—
	2:6-Dideoxy-D-allose (D-digitoxose)	—	—
Uronic acid	D-Glucuronic	—	—
	D-Glucurono-δ-lactone	—	—
	D-Galacturono-δ-lactone	—	—
Aldosamine	D-Glucosamine	—	—
	N-Acetyl-D-glucosamine	—	—
Aldose ester	D-Glucose 6-phosphate	—	—
Aldose ether	3-*O*-Methyl-D-glucose	—	—
	2:6-Dideoxy-3-*o*-methyl-D-*lyxo-hexose* (D-diginose)	—	—
	2:6-Dideoxy-3-*o*-methyl-D-*ribo-hexose* (D-cymarose)	—	—
	6-Deoxy-3-*o*-methyl-D-galactose (D-digitalose)	—	—
	6-Deoxy-3-*o*-methyl-D-glucose (D-thevetose)	—	—
Disaccharide	Trehalose	—	Maltose (0·38)
	2-β-Fructosylglucose	—	Cellobiose (0·37)
	3-β-Fructosylglucose	—	Lactose (0·35)
	Laminaribiose	—	Melibiose (0·32)
	Turanose	—	6-β-Fructosylglucose (0·56)
	Leucrose	—	Gentiobiose (0·35)
	Inulobiose	—	Isomaltose (0·35)
	Levanbiose	—	—
Trisaccharide	—	—	Maltotriose (0·19)
	—	—	Panose (0·17)
	—	—	Isomaltotriose (0·15)
	—	—	Manninotriose (0·13)
Homologous polymer series	—	—	Oligodextrans of DP§ 2–7
	—	—	Oligoamyloses of DP 2–7
	—	—	Oligoxylans of DP 2–6

* Ketoses as well as aldoses are listed, since a ketose could be regarded for the purposes of the present classification as an aldose in which CH_2OH had replaced H at C-1.

† In addition to arabinosylfructoside, a β-fructofuranosylarabinose ($R_{sucrose}$ 1·24) was formed in this system. The yield of the fructosylarabinose exceeded that of the arabinosylfructoside.

‡ A fructosylsorbose ($R_{sucrose}$ = 0·96) was formed in good yield.

§ DP, Degree of polymerization.

Table 3. *Influence of specific alterations in atomic arrangement of aldose on the acceptor activity of* C-1

Carbon site of the alteration in aldose	Nature of alteration	Example (active parent compound→tested product)	Influence of alteration on acceptor activity of C-1*
1	CH_2OH for H on C	D-Glucose→D-glucoheptulose	−
	Glucosyl for H in OH	D-Glucose→trehalose	−
	CH_3 for H in OH	D-Glucose→methyl α-D-glucoside	−
2	Reduction	D-Glucose→2-deoxy-D-glucose	−
	Epimerization	D-Galactose→D-talose	−
	NH_2 for OH	D-Glucose→D-glucosamine	−
	Acetylamino for OH	D-Glucose→*N*-acetyl-D-glucosamine	−
	Hexosyl for H in OH	D-Glucose→2-β-fructosylglucose	−
3	Inversion	D-Xylose→D-ribose	−
	CH_3 for H in OH	D-Glucose→3-*o*-methyl-D-glucose	−
	Glucosyl for H in OH	D-Glucose→laminaribiose	−
4	Inversion	D-Glucose→D-galactose	+
	Glucosyl for H in OH	D-Glucose→lactose	+
5	Inversion	D-Altrose→L-galactose	+
	CH_2OH for H on C	D-Xylose→D-glucose	+
6	Reduction	L-Galactose→L-fucose	+
	Oxidation	D-Glucose→D-glucuronic acid	−
	Hydroxyalkyl for H on C	D-Galactose→D-glycero-D-galacto-heptose	+
	Phosphoryl for H in OH	D-Glucose→D-glucose-6-phosphate	−
	Glycosyl for H in OH	D-Glucose→isomaltose	+
2 and 3	Inversion at each C atom	D-Glucose→D-altrose	±
2 and 4		D-Glucose→D-talose	−
3 and 4		D-Glucose→D-gulose	−
2, 3 and 4		D-Xylose→L-xylose	±
2, 3, 4 and 5		D-Glucose→L-glucose	±
3 and 6	Oxidation with ring closure	D-Glucose→D-glucuronolactone	−

* +, Product has the same order of acceptor activity as the parent; ±, product exhibits much less acceptor activity than the parent; −, product, in contrast to parent, lacks acceptor activity.

A classified list of the aldoses that have been examined is in Table 2. Appropriate examples of the effect exerted by local alterations of atom arrangement in a parent aldose on the acceptor activity of C-1 are collected in Table 3. Additional examples can be derived from Table 2.

The findings underlying the above-given aldose classification were obtained by us in repeated experiments which involved three separate batches of the enzyme. However, in experiments carried out on another enzyme batch, Mr I. Abramovici, in this Laboratory, observed that the aldopentoses (L-xylose, D-arabinose) which had formerly been found within class (*b*) showed reactivities similar to those of members of class (*c*).

All the chromatographically resolved members of three homologous polymer series of oligoaldoses (partial hydrolysates of dextran, glycogen and xylan) accepted fructose at C-1 of the reducing-end unit. The reaction was effected readily, irrespective of the lengths of the polymer chains. Plots of log α' against degree of polymerization (French & Wild, 1953) are shown in Fig. 1.

Oligofructoses [degree of polymerization (DP), 2–8] prepared from partial acid hydrolysates of both inulin and levan all failed to accept fructose at C-2 of the reducing-end unit. On the other hand, carbinol sites in some oligolevans are known to be active acceptors (Mattoon, Holmlund, Schepartz, Varva & Johnson, 1955; Avigad, 1956; Dedonder & Péaud-Lenoël, 1957; Péaud-Lenoël, 1957*b*). This aspect will be the subject of a separate communication.

With one exception (L-sorbose), the compounds in classes (*a*) and (*b*) failed to inhibit either levan or fructose production to an extent evident chromatographically (>50%). On the other hand, the compounds in class (*c*) all did produce such an effect and also noticeably inhibited the formation of 1^F-β-fructosylsucrose. Accordingly, it can be inferred that the members of classes (*a*) and (*b*) possess a markedly lower affinity for the enzyme than any member of class (*c*).

Although L-sorbose did not accept fructose at C-2, it contained a carbinol site which was a good acceptor. This ketose thus exerted a marked inhibitory effect both on levan and fructose formation. The fructosylsorbose so formed reduced alkaline triphenyltetrazolium chloride. On this basis (Feingold *et al.* 1956), it has been inferred that the position of substitution in L-sorbose was not C-1.

Donor activity among aldosylketosides

It was known from a previous investigation (Hestrin *et al.* 1956) that donor activity is common to several aldosyl β-fructofuranosides which contain an unsubstituted *trans*-disposed OH pair at C-2 and C-3 of the aldose moiety. It has now been shown that additional compounds in this class show the same behaviour, namely, β-L-arabinosyl β-fructofuranoside (L-arabsucrose), D-arabinosyl β-fructofuranoside (D-arabsucrose), 4^G-β-galactosylsucrose (lactsucrose) and 4^G-α-glucosylsucrose (maltsucrose). On the other hand, 2^G-α-galactosylsucrose (umbelliferose; Wickstrom & Baerheim-Svendsen, 1956) was not split by levansucrase. The rate of the enzymic decomposition of 0·2 M-[^{14}C]-sucrose was not decreased by 0·2 M-umbelliferose (inhibition < 5 %). Thus it can be suggested that in sucrose, as in free glucose, ability to react with levansucrase was abolished by introduction of a hexosyl group at C-2 of the glucose moiety. The diazouracil reaction (Raybin, 1933) of umbelliferose was positive.

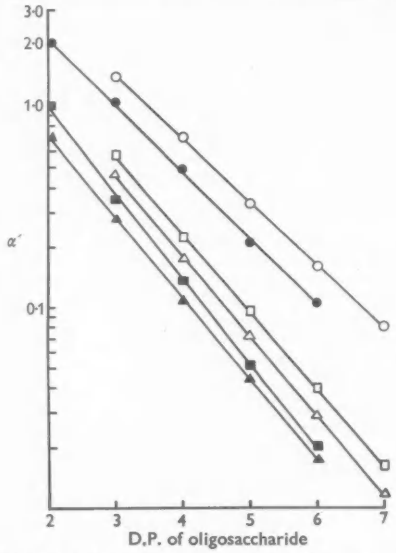

Fig. 1. Transfer of fructose from sucrose to C-1 of terminal reducing-end unit in homologous polymer series of oligoaldoses. Reaction mixtures containing 6 % (w/v) of reducing oligosaccharide and 1·5 % (w/v) of sucrose were incubated with levansucrase at pH 5·4 for 8 hr. at 37°. Chromatograms were developed with solvent *a* (Table 1). ▲, ■, ●, Homologous polymer series (reducing aldoses) prepared from dextran, glycogen and xylan respectively by partial acid hydrolysis. △, □, ○, Corresponding aldosylfructoside series formed from the polymer series. $\alpha' = R_{glucose}/(1 - R_{glucose})$ (French & Wild, 1953).

It was known from the previous study mentioned that introduction of a hexosyl group into the fructosylic moiety of sucrose at C-1, C-3 or C-6 abolishes donor activity. However, the question whether configurational changes in the ketose moiety of sucrose are able to alter the donor activity had still to be decided. Among disaccharides which differ from sucrose only in the ketose moiety at single carbons, two compounds (both synthesized by sucrose phosphorylase) have now been investigated: α-D-glucopyranosyl α-L-sorbofuranoside (an epimer of sucrose at C-5 of fructose) (Hassid, Doudoroff, Barker & Dore, 1945) and α-D-glucopyranosyl D-rhamnulofuranoside (6^F-deoxysucrose) (Palleroni & Doudoroff, 1956). Neither of these compounds was cloven by levansucrase.

Quantitative description of a typical example of the aldosyl-exchange reaction (eqn. 2)

As examples of the aldoses assigned to class (*c*), melibiose and glucose were chosen for quantitative study. Initial reaction rates in systems of donor (sucrose, raffinose) with and without exogenous added acceptor (melibiose in the system with sucrose, glucose in the system with raffinose) are compared in Table 4. Time courses are detailed in Figs. 2–4.

Table 4. *Initial relative rates of transfer of fructose to competing acceptors*

Initial reaction rates are calculated from measurements made within the first 2 hr. of reaction and are expressed on a molar basis in relative units. During the interval of observation, the measured rates remained substantially constant. Sucrose and glucose concn. was 0·2 M; raffinose and melibiose concn. was 0·4 M.

Component measured	[^{14}C]Sucrose only	[^{14}C]Sucrose + melibiose	Raffinose + [^{14}C]glucose	[^{14}C]Sucrose + raffinose*	Raffinose only
Sucrose	−100	−73	+13	−71	—
Raffinose	—	+46	−35	< +1	−50
Glucose	+97	+71	−14	+69	—
Fructose	+52	+14	+11	+37	+21
Anhydrofructose (levan)	+46	+16	+14	+30	+26
1^F-β-Fructosylsucrose	+2†	+1	—	+1	—

* Rates in this column refer only to the ^{14}C-labelled components formed from [^{14}C]sucrose in the presence of both sucrose and raffinose.

† Oligosaccharides other than those whose formation is reported were formed at rates <1.

The data confirm an earlier evaluation (Hestrin *et al.* 1956) of the relative order of the competitive powers of water and carbinol groups in sucrose. It is further noteworthy that the rates of transfer of fructose from sucrose or raffinose to C-1 of aldose (glucose, melibiose) were greater than was the rate of production of levan. Findings that will be detailed elsewhere have indicated that a many times slower transfer of fructose to C-1 of aldose can be effected by this enzyme from levan as donor. However, to demonstrate such activity on the part of levan, a degraded levan preparation had to be used as donor, and relatively high concentrations of both aldose and enzyme were maintained. The notion that the transfer of fructose from sucrose to C-1 of aldose occurs necessarily via levan (Péaud-Lenoël, 1957*a*) can be refuted on the basis of the above observations.

Decomposition of sucrose both in the system without any added aldose (Fig. 2) and in the system with added melibiose (Fig. 3) showed an abrupt intervening decrease in rate. This feature can be ascribed at least in part to an action of glucose formed during the reaction. Glucose competes with sucrose for the enzyme (Fig. 5) and exerts an inhibitory influence which appears to be even greater than that of melibiose (Table 4).

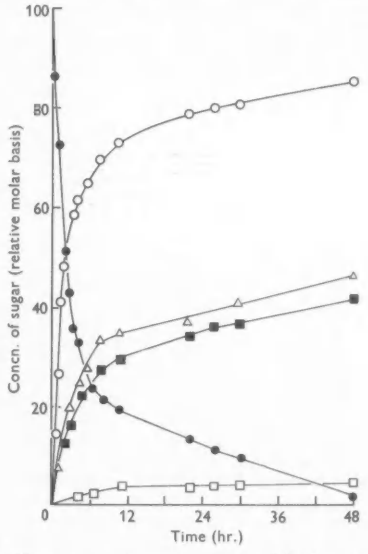

Fig. 2. Time course of reactions initiated by levansucrase acting on 0·2 M-[^{14}C]sucrose. ●, Sucrose; ○, glucose; ■, levan; △, fructose; □, 1^{F}-β-fructosylsucrose. Concentrations are expressed on a relative molar basis for sugars, and in moles of anhydrofructose for levan, the initial sucrose concentration being taken as 100.

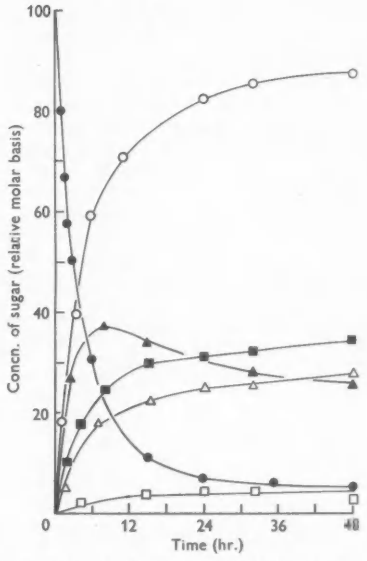

Fig. 3. Time course of reactions initiated by levansucrase acting on 0·2 M-sucrose and 0·4 M-melibiose. ▲, Raffinose; for explanation of other symbols see Fig. 2. Concentration is expressed as in Fig. 2.

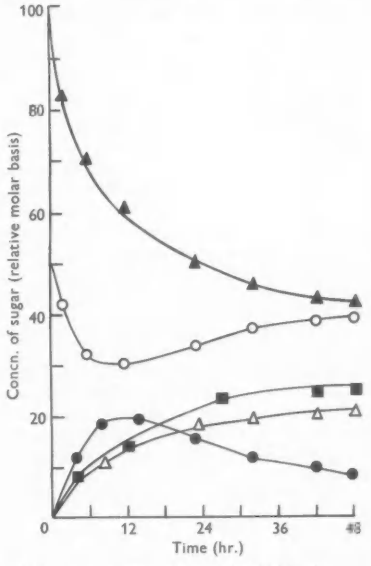

Fig. 4. Time course of reactions initiated by levansucrase acting on 0·4 M-raffinose and 0·2M-[^{14}C]glucose. For explanation of symbols and ordinate see Figs. 2 and 3.

In good agreement with the assumption that different donor systems are activated by an identical enzyme, the decomposition of one donor (sucrose) was inhibited by an alternate donor (raffinose) (Table 4). Melibiosyl fructoside (i.e. raffinose) and free melibiose inhibited decomposition of sucrose about equally.

The rate of the incorporation of ^{14}C of sucrose into raffinose was found to be negligibly low at zero time in a substrate system consisting initially of only sucrose and raffinose (Table 4), and to increase slowly with progressing decomposition of donor. This behaviour has suggested that aldosyl radicals in an aldosyl fructoside pair cannot be exchanged directly by the levansucrase system.

Effects of concentration of aldoses and donor on the reaction velocity conformed to the Michaelis-Menten relation (Fig. 6). It was noted that the formations of fructose and levan both responded equally to an alteration in substrate concentration (sucrose, glucose) throughout the investigated ranges. The values of the Michaelis constants (K_m) of glucose (as acceptor at C-1) and sucrose (as donor) were very close to one another, being found to be 0·074 M and 0·062 M respectively. K_I of glucose, too, was a similar value, namely 0·072 M (Fig. 5), the value found being the same irrespective of whether decomposition of sucrose or formation of levan served as the basis of the calculation. On the other hand, K_m of melibiose (as acceptor at C-1) was a relatively high value, 0·42 M. Since K_m of sucrose and glucose are similar, one might anticipate K_m of raffinose to be like that of melibiose. However, K_m of raffinose has not as yet been measured. K_m of sucrose in *Bacillus subtilis* levansucrase has been found to be 0·09 M (Péaud-Lenoël, 1957*b*).

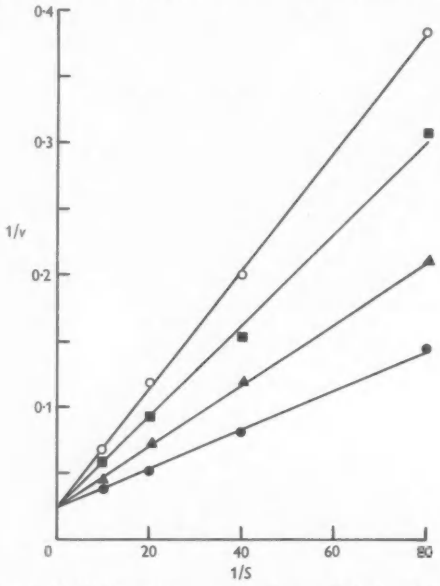

Fig. 5. Inhibition of levansucrase by methyl glycosides and glucose. Reaction mixtures contained varying concentrations of [^{14}C]sucrose in the presence of 0·1 M-methyl glycoside or -glucose. Samples (10 μl.) whose activity was 166 μmc were taken at 120 min. Within this time, reaction velocities remained practically constant in every case. In the experiment with added glucose, the net increase of glucose (Δglucose) was calculated as the sum (moles): levan anhydrofructose + fructose + 1^F-β-fructosylsucrose. In this system the amount of [^{14}C]-glucose which had been liberated from sucrose by exchange of the latter with free glucose was calculated from the difference (A = counts/min.): A (glucose) − A (Δglucose). The amount of this exchange was equivalent to approx. 15% of the sucrose found at 120 min. in the mixture. S = Molarity of sucrose; v = velocity of decomposition of sucrose (μmoles/ml./2 hr.). ●, Sucrose without added inhibitor; ▲, with methyl α-D-glucopyranoside; ■, with methyl β-D-fructofuranoside; ○, with D-glucose.

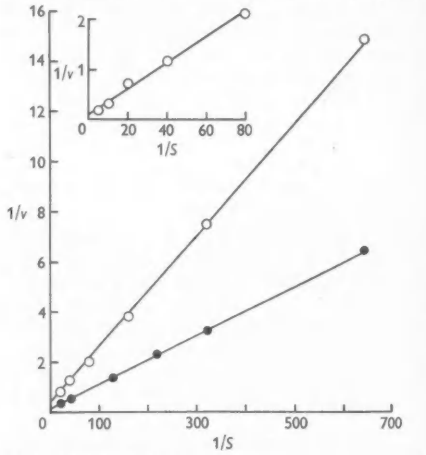

Fig. 6. Michaelis constants of levansucrase in respect of glucose, melibiose and sucrose. S = Concn. (molarity) of a varied component (glucose, melibiose or sucrose, as specified below) of substrate system. v = Reaction velocity in μmole/ml./t, where t is reaction time (min.) as specified. Within such times, all the reaction rates remained steady. Main figure: ●, sucrose formation at t = 90 in a reaction system of 0·2 M-raffinose with [^{14}C]-glucose, the concn. of the latter being varied; ○, decomposition of sucrose at t = 10 in a reaction system of sucrose without any added aldose, the concn. of sucrose being varied. Inset: ○, formation of raffinose at t = 120 in a reaction system of 0·1 M-[^{14}C]sucrose and melibiose, the concn. of the latter being varied.

Sucrose–raffinose systems attained the same 'steady state' whether sucrose or raffinose was the primary donor. The value of the molar quotient ([raffinose] [glucose]/[sucrose] [melibiose]) in these systems approached 2·2 (Fig. 7). If this value is taken as a true Nernst equilibrium constant (K), the loss of free energy ($-\Delta F'$) attending transfer of fructose from sucrose to the anomeric carbon position of α-melibiose can be estimated from the relationship $\Delta F' = -RT \cdot \ln K$ to be 0·48 kcal. (37°; pH 5·4), i.e. a small value consistent with the placement of both sucrose and raffinose on nearly the same energy level, somewhat below that of adenosine triphosphate (Robbins & Boyer, 1957; Vladimirov, Vlassova, Kolotilova, Lyzlova & Panteleyeva, 1957; cf. review by Mahler, 1957).

Time courses and equilibrium values of levansucrase-catalysed fructose transfer to aldose C-1 in several other donor–acceptor systems (sucrose–cellobiose, sucrose–maltose, raffinose–D-xylose, raffinose–L-xylose and raffinose–D-arabinose) have been determined by analogous methods in experiments carried out jointly with Mr I. Abramovici and to be detailed elsewhere. In all these systems, the reaction followed a more or less similar time course and values of K were in the range 1–2.

Inhibition of levansucrase by methyl β-D-fructofuranoside (Me β-fr)

Fructosides in which C-2 of fructose is linked to a carbinol carbon (R < fr compounds) are not split at the glycosidic linkage by levansucrase (Hestrin & Avineri-Shapiro, 1944; Hestrin *et al.* 1956). In the course of the present work, the behaviour of a pure preparation of Me β-fr, a simple example of this class, was examined in some detail.

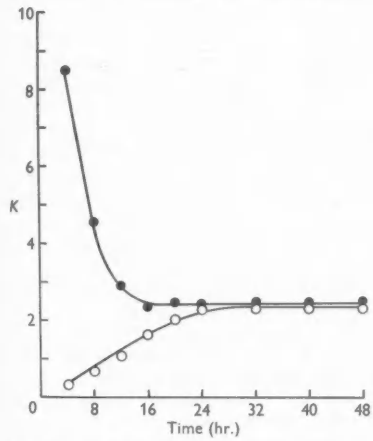

Fig. 7. Approach of a levansucrose–sucrose–raffinose system to apparent equilibrium. Curves are plotted from the data given in Figs. 3 and 4. K = [raffinose] [glucose]/[sucrose] [melibiose], the concentrations being expressed in moles. ○, Interaction of sucrose with melibiose; ●, interaction of raffinose with glucose.

Me β-fr did not serve as a donor with levansucrase though it was formed by the same enzyme in a reaction system consisting of methanol as acceptor and sucrose as donor. Mould fructosylases, on the other hand, both form and cleave Me β-fr (Schlubach & Rauchalles, 1925; Bealing, 1953; Edelman, 1954, 1956; Breuer & Bacon, 1957).

Decomposition of sucrose by levansucrase was found to be inhibited competitively by Me β-fr. In contrast, several other compounds of the type of R < fr, namely levanbiose and inulobiose (in which R is fructose) and 1^F-β-fructosylsucrose (in which R is sucrose), failed to inhibit decomposition of sucrose by levansucrase, under the standard conditions of test, to a chromatographically evident extent (> 50 %). Evidently the nature of the afructon group was exerting a marked effect on the ability of the β-fructofuranosyl radical to enter into combination with the active surface of the enzyme. Free fructose, even at a concentration as high as M, failed to inhibit decomposition of sucrose appreciably. The value of K_I of Me β-fr, as estimated on the basis of measurements of sucrose decomposition, was 0·085 M (Fig. 5), a value only slightly higher than K_m of sucrose.

Me β-fr retarded formation of levan more markedly than it did the formations of fructose and 1^F-β-fructosylsucrose (Table 5). To account for this disparity the possibility can be considered that, in addition to an ability to combine with the enzyme at the sucrose-binding site, Me β-fr is able to combine with the enzyme at a levan-binding site (cf. Péaud-Lenoël, 1957*b*). It can be seen in Table 5 that transfer of fructose to Me β-fr resulted in the formation of a methyl β-fructobioside. At equal acceptor concentration (0·2 M), the amount of the transfer of fructose to Me β-fr was almost twice that to sucrose. The following relationship applied (moles): sucrose decomposed with Me β-fr/sucrose decomposed without Me β-fr = fructose formed with Me β-fr/fructose formed without Me β-fr = (levan anhydrofructose formed with Me β-fr + methyl β-fructobioside)/levan anhydrofructose formed without Me β-fr. In other words, the excess of inhibitory effect of Me β-fr on production of levan, i.e. the difference between the extent of the inhibition of production of levan and that of the inhibition of production of fructose, was balanced by the amount of the transfer of fructose to Me β-fr.

The values of $R_{sucrose}$ of 1^F- and 6^F-β-fructosylsucrose have been found to be respectively 0·55 and 0·44 (in solvent *b*) (Feingold *et al.* 1956). The ratio

Table 5. *Influence of methyl β-D-fructofuranoside* (Me β-fr) *on transfer reactions catalysed by levansucrase*

Systems contained 0·2 M-[^{14}C]sucrose in the presence of stated concentrations of Me β-fr. The activity of portions (10 μl.) taken for analysis was 166 μmc.

Component	Rate of formation of component* (μmoles/ml./hr.)		Inhibition of formation of component (%)	
	Without Me β-fr	0·18 M-Me β-fr	0·18 M-Me β-fr	0·36 M-Me β-fr
Levan	7·20	2·50	65	76
Difructosylsucrose	0·13	0·07	46	—
1^F-β-Fructosylsucrose	1·28	0·78	39	64
Glucose†	12·60	7·60	40	56
Methyl β-fructobioside†	—	1·90	—	—
Fructose	3·56	2·22	38	54

* Values are calculated on the yields observed at 4 hr. From yields observed at 6 hr. values about 15% lower were found.

† $R_{sucrose}$ of methyl β-fructobioside was 1·37 (solvent *b*), i.e. a value close to that of glucose. To estimate the rate of formation of methyl β-fructobioside, the following theoretically established relationship was used (A = counts/min.): A (methyl β-fructobioside) = $\frac{1}{3}$ ($A_g - A_s$), where A_g is the radioactivity of the glucose area (i.e. the area which contains glucose and methyl β-fructobioside), and A_s is the sum: A (levan) + A (fructose) + $\frac{1}{3}$ A (1^F-β-fructosylsucrose) + $\frac{1}{2}$ A (difructosylsucrose). The amount of glucose was calculated from the difference: $A_g - A$ (methyl β-fructobioside).

of the chromatographic mobility of methyl β-fructobioside to that of Me β-fr in the same solvent system was 0·54. This suggests that fructo-C-1 in Me β-fr was the active acceptor site, i.e. that the preferred reaction sites both in Me β-fr and sucrose correspond to each other.

Inhibition of levansucrase by methyl α-D-glucopyranoside (Me α-gl)

The ability of an α-glucoside to combine with the active surface of levansucrase was found to be markedly influenced by the nature of the aglycon group. Thus Me α-gl (aglycon = CH_3) was an inhibitor of levan formation (Hestrin & Avineri-Shapiro 1944), yet trehalose (aglycon = α-glucosyl) and 1^F-β-fructosylsucrose (aglycone = β-difructofuranosyl) failed to inhibit formation of either levan or fructose noticeably in the standard conditions of reaction. In good agreement with the assumption that glucosyl in Me α-gl combines with the enzyme at a sucrose-binding site, it was found that the action of Me α-gl is competitive in respect of sucrose (Fig. 5). K_I of Me α-gl is 0·18 M.

Although Me α-gl combined with the enzyme the glucosidic linkage in Me α-gl was not split. Carbinol sites in Me α-gl, as those in free glucose, were all poor acceptors of fructose.

DISCUSSION

Several families of polymers consisting of chains of monosyl residues terminated by fructose in the sucrose configuration are known: 'raffinoses' in seeds (Herrisey, Fleury, Wickstrom, Courtois & LeDizet, 1954), 'maltsucroses' among the products formed by Schardinger enzyme (French & Wild, 1953) and in honeydew (Wolf & Ewart, 1955) and 'dextransucroses' in a sucrose culture of a *Streptococcus* (Hehre, 1956). It is the commonly held view and one which has been supported experimentally that such polymers can be generated by processes of repetitive transglycosylation initiated to an appropriate carbinol site in sucrose. However, results here described suggest that the catalysed transfer of fructose to C-1 of aldoses (eqn. 1) may represent still another means by which monosyl chains bearing a fructose terminal are formed in nature.

Synthesis of sucrose by a non-enzymic and unequivocal method has been accomplished recently (Lemieux & Huber, 1956). The same method could no doubt be applied to syntheses of sucrose analogues (R ~ fr). Presumably, however, such syntheses could be accomplished more conveniently in respect of a wide, now-defined range of R with the help of levansucrase. This enzyme is specific as between anomers of an aldose. Moreover, the transfer of a fructose group from donor to an acceptor is uniformly effected by this enzyme with net retention of fructose configuration. Thus only a single aldosyl fructoside (a product conforming in structure and configuration to sucrose) can be expected to be formed from any given aldose on its reaction with a fructose donor in the presence of levan sucrase.

On the basis of the present survey of many aldoses, the following generalizations concerning the effect of atomic arrangements on the acceptor activity of C-1 of aldose are presented:

(1) Among neutral unsubstituted aldopyranoses, those with configuration conforming at C-2 and C-3 to threose (D- or L-) exhibit activity, whereas those with configuration conforming to erythrose (D- or L-) lack activity.

(2) Activity is consonant with wide variation in atomic arrangement in aldose at C-4 and other atoms remote from C-1 to C-3. However, a carboxyl group on C-5 or a phosphoryl group at C-6 in aldose suppresses the activity. This behaviour is consistent with the assumption that a negative charge occurs in the enzyme surface and that the approach of an anionic aldose is thereby hindered.

(3) Activity is lacking in aldopyranoses in which the C-2 hydroxyl group has been replaced by H or

NH_2 or in which the C-2 or C-3 hydroxyl group is substituted (glycosyl, CH_3) or in which the C-1 hydrogen atom has been replaced by $-CH_2OH$.

Thus the suggestion can be made that the C-2 and C-3 hydroxyl groups play an active part in the mechanism of the interaction of levansucrase with an aldose.

In any hypothesis concerning the structure of the active surface of levansucrase, account must be taken of some general conclusions which may be derived from the present study. (*a*) Donor activity of sucrose is abolished if alterations of atomic arrangement or composition are made at 'sensitive' carbon sites either in the glucose or fructose moiety of the disaccharide, e.g. at gluco-C-2 or at fructo-C-5 and C-6. (*b*) Acceptor activity of C-1 is confined to aldopyranoses which possess an unsubstituted *trans*-disposed hydroxyl pair at C-2 and C-3 (cf. also, Péaud-Lenoël, 1957*a*). (*c*) The ability of glycosides containing either α-glucopyranosyl or β-fructofuranosyl as glycon to inhibit decomposition of sucrose is confined to those glycosides whose aglycon group satisfies a rigid specificity requirement. Inhibition was shown to be produced, for example, by appropriate methyl glycosides but not by several corresponding glycosyl glycosides. To account for the above properties of levansucrase, it is suggested that for an aldosyl fructoside to be attacked by the enzyme the active surface of the latter must establish close contact with several hydroxyl groups in substrate regions on both sides of the glycosidic oxygen. This hypothesis implies that the glucose moiety of sucrose is bound by an aldophilic region and that the fructose moiety is bound by a neighbouring fructophilic region of the surface of the enzyme.

Since a levansucrase system catalyses several kinds of fructose-transfer reactions, the question arises whether these reactions are all effected by the same catalyst. It has been shown above that both the transfer of fructose from donor to water (fructose formation) and that to anhydrofructocarbinol (levan formation) exhibit an equal response to alteration of the concentration of sucrose. Similarly, they have also been found to be retarded equally by an added aldose (glucose, melibiose). Furthermore, it has been shown that the following are able to enter into competition within a levansucrase system (Avigad, 1955; Hestrin *et al.* 1956; Péaud-Lenoël, 1957*b*; present results): (1) water, fructo-C-1 carbinol of sucrose, carbinols of levan, C-1-carbonyl of glucose and C-1-carbonyl of some other aldopyranoses (as acceptors); (2) sucrose and raffinose (as donors). Resolution of levansucrase into separate donor- and acceptor-activating systems has not proved possible so far. The results in hand are thus all in agreement with the assumption that donor and acceptors are activated in a levansucrase system by a single protein (Hestrin *et al.* 1956; Péaud-Lenoël, 1957*b*).

SUMMARY

1. Neutral unsubstituted aldopyranoses conforming configuratively to L-threose and D-threose at C-2 and C-3 formed aldosyl fructoside when incubated with a donor of fructose in the presence of *Aerobacter levanicum* levansucrase. Acceptor activity of C-1 in a free aldose was suppressed by any one of the following: epimerization at C-2, inversion at C-3, introduction of a substituent (glycosyl, CH_3) at C-2 or C-3; deoxygenation at C-2; replacement of hydrogen at C-1 by $-CH_2OH$. An anionic group in an aldose suppressed the activity of C-1. Other wide variations in atomic arrangement at C-4 and other atoms remote from C-1 to C-3 in free aldose did not suppress acceptor activity of C-1.

2. Reversible transfer of fructose from sucrose to C-1 of α-melibiose or some other aldoses proceeded with small loss of free energy ($-\Delta F' =$ approx. 0·5 kcal.).

3. Values of K_m for levansucrase in respect of glucose and melibiose (as acceptors at C-1) and in respect of sucrose (as donor) were 0·074, 0·42 and 0·062 M respectively.

4. Methyl β-D-fructofuranoside and methyl α-D-glucopyranoside competitively inhibited the decomposition of sucrose by levansucrase. Their K_I values were 0·085 and 0·18 M respectively.

5. Donor activity in sucrose was abolished by any one of the following: introduction of a glycosyl group at gluco-C-2 or at fructo-C-1, -C-3 or -C-6; inversion of configuration at fructo-C-5; deoxygenation at fructo-C-6.

6. The observed substrate and inhibitor specificities of levansucrase suggest the hypothesis that the active surface of this enzyme contains a fructophilic region alongside an aldophilic region and that the catalytic mechanism depends on an interaction of each of these regions with several hydroxyl groups of the substrate.

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The Sedimentation Characteristics of Deoxyribonucleic Acid from Normal and Diseased Human Tissues

By E. E. POLLI

Istituto di Clinica Medica Generale e di Terapia Medica, Universita de Milano, Via Francesco Sforza, 35, Milano

and K. V. SHOOTER

Chester Beatty Research Institute, Institute of Cancer Research, Royal Cancer Hospital, London

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Clinical investigations of certain anti-metabolites which interfere with metabolism of nucleic acid have shown that they act in different ways upon the leucocytes present in the bloodstream in myeloid and lymphatic leukaemia. This suggests that the nucleic acids of these leucocytes may differ. The physical properties and chemical composition of the deoxyribonucleic acid from normal and leukaemic human leucocytes have been investigated by Polli (1957) and Polli & Semenza (1955, 1956). It was found that the effect of urea and of alkali on the viscosity of solutions of deoxyribonucleic acid prepared from myeloid leukaemia leucocytes was different from that of solutions of deoxyribonucleic acid from normal or lymphatic leukaemia leucocytes. Differences be-

tween the electrometric-titration curves of these preparations were also found (Polli, Di Majorca, Rosoff & Cavalieri, 1958). These results indicate that the deoxyribonucleic acid samples differ in their resistance to denaturation (see Rice & Doty, 1957). Since the base composition of the samples from all these sources was found to be the same, it would appear that the variations in the ease with which the samples can be denatured are due to differences in the physical structure of the molecules. In an attempt to determine the nature of these differences Polli & Fasoli (1956) studied the sedimentation characteristics of a variety of samples of deoxyribonucleic acid, including preparations from leucocytes of myeloid leukaemia. The results of their experiments, which were performed with concentrations in the range 0·22–0·01%, failed to indicate any differences between the samples. It was, however, appreciated that at the lowest concentration used considerable interaction between the molecules still occurs and that this might obscure possible differences. Sedimentation experiments have now been performed at lower concentrations and the results obtained show significant differences between the samples of deoxyribonucleic acid from normal and leukaemic leucocytes.

EXPERIMENTAL

Three of the samples were prepared by the method of Gulland, Jordon & Threlfall (1947) as modified by Chargaff (1955). The remainder were prepared by the method of Kay, Simmons & Dounce (1952) with the modifications suggested by Chargaff (1955) (see Table 1). During the course of the preparation 0·01 M-NaCl was used instead of water as a solvent to prevent denaturation. For the experiments to be reported solutions were obtained by dissolving the deoxyribonucleic acid (DNA) in 0·01 M-NaCl and then adding an equal volume of 0·4 M-NaCl. These solutions were then further diluted with 0·2 M-NaCl as required. No attempt was made to maintain a constant pH in these experiments, since Alexander & Stacey (1955) have shown that the shape of DNA molecules changes very little in the range pH 4–7. The sedimentation experiments were performed in a Spinco analytical ultracentrifuge fitted with an ultraviolet-light-absorption optical system. From the results obtained at a series of concentrations in the range 0·01–0·001% of DNA, the curves giving the distribution of sedimentation coefficients were calculated in the manner described by Shooter & Butler (1956). From these curves the curves giving the distribution of sedimentation coefficients at infinite dilution were obtained by the method of Oth & Desreux (1955).

It has been found that with calf-thymus DNA the presence of aggregates cross-linked by small amounts of residual protein may profoundly affect the shape of the distribution curve. Treating the samples with chymotrypsin considerably reduced the proportion of such aggregates (Butler, Phillips & Shooter, 1957). To determine whether the different shapes of the distribution curves observed in the present work were due to the presence of varying amounts of protein-linked aggregates, the effect of treating some of the samples with chymotrypsin was investigated. Solutions of samples 1–10 in 0·2 M-NaCl were prepared at four different concentrations, and each solution was divided into two parts. To one part of each a small amount of crystalline chymotrypsin was added. The solutions were shaken to dissolve the enzyme and were then stored at 4° for 1–3 days (these conditions were the same as those used in the experiments with calf-thymus DNA). Sedimentation experiments were performed with each of these solutions and the sedimentation-coefficient distribution curves were calculated. It was found that in all cases the distribution curves observed in the presence and in the absence of the enzyme were identical.

RESULTS

The curves giving the distribution of sedimentation coefficients at infinite dilution of the 16 samples of DNA investigated are shown in Figs. 1–5; some of the characteristics of these curves are given in Table 1. The curves in the figures are the integral distribution curves: the ordinate corresponding to a sedimentation coefficient S_0 gives the fraction of DNA with sedimentation coefficient $\leqslant S_0$, and the slope of the line at the point gives the weight fraction of DNA with sedimentation coefficient S_0. It can be seen that the samples of DNA from the leucocytes of lymphatic leukaemia prepared by the method of Gulland *et al.* (1947) (Fig. 1, *A*) are quite different from those prepared by the method of Kay *et al.* (1952) (Fig. 1, *B*). The liberation of DNA from calf-thymus nucleoprotein with these two methods has been studied by Shooter & Butler (1957). It was found that when chloroform was used to denature and remove protein (Gulland *et al.* 1947) a marked fractionation of the DNA occurred, much of the material with high rate of sedimentation being lost. When, however, the protein was removed from the nucleoprotein complex with dodecyl sulphate (Kay *et al.* 1952) the distribution curve of the product was almost identical with that of the DNA in the original nucleoprotein. The method of Gulland *et al.* cannot therefore be regarded as reliable. The results obtained for the three samples of DNA from leucocytes of lymphatic leukaemia with the method of Gulland *et al.* will not therefore be considered further in the discussion.

It can be seen from the figures that, except for the samples from leucocytes of myeloid leukaemia (Fig. 2), the reproducibility of the preparations from each source is good. The curves for the different samples from normal leucocytes (Fig. 3) and the leucocytes of lymphatic leukaemia (Fig. 1) do not differ by more than $\pm 1{\cdot}2$s over the whole range of the distributions. The two samples from non-leukaemic spleen, 4MD and 5MD (Fig. 4), show even better agreement, the curves differing by

less than $\pm 0{\cdot}5$s. The distribution curves for preparations from myeloid leukaemia leucocytes differ by as much as ± 3s (Fig. 2).

DISCUSSION

If the weight average sedimentation coefficients of the different samples (Table 1) are compared, it is found that for deoxyribonucleic acid from normal leucocytes $S^0_{av.} = 26{\cdot}8$–$27{\cdot}0$s; preparations from leucocytes of lymphatic leukaemia (excluding samples 3, 4 and 6, see above) give higher values, $S^0_{av.} = 28{\cdot}4$–$30{\cdot}8$s, and preparations from leucocytes of myeloid leukaemia, with the exception of sample 10, give lower values, $S^0_{av.} = 22{\cdot}8$–$27{\cdot}0$s. These results indicate that it is in general possible to distinguish between normal and leukaemic leucocytes in terms of the sedimentation characteristics of the deoxyribonucleic acid. Two problems are raised: (*a*) what is the significance of the differences between normal and leukaemic deoxyribonucleic acid and (*b*) why do preparations from leucocytes of myeloid leukaemia exhibit a lack of reproducibility whereas samples from the other sources studied are reproducible?

Histological investigations (Wintrobe, 1956*a*) have shown that in chronic lymphatic leukaemia there is a striking uniformity of white cells in the blood, small lymphocytes accounting for 90–99% of the total cells. In chronic myeloid leukaemia, on the other hand, the leucocytes in any one sample exhibit varying degrees of maturity. Myeloblasts, myelocytes, metamyelocytes and polymorphonuclear leucocytes are usually found in this latter disease, the proportions of the different cells varying from patient to patient. The preparations of

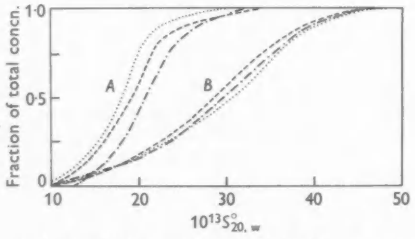

Fig. 1. Sedimentation-coefficient distribution curves at infinite dilution for DNA from lymphatic leukaemia leucocytes. Group *A*: prepared by the method of Gulland *et al.* (1947); · · ·, 2; – – –, 4; —·—, 6. Group *B*: prepared by the method of Kay *et al.* (1952); · · ·, 1; —·—, 3; – – –, 5.

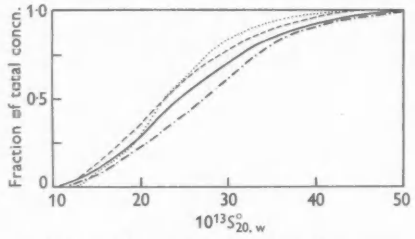

Fig. 2. Sedimentation-coefficient distribution curves at infinite dilution for DNA from myeloid leukaemia leucocytes: —, 7; – – –, 8; · · ·, 9; —·—, 10. 10 was from a patient before treatment, 7 from the same patient after treatment with Myleran; 8 and 9 were from two other patients.

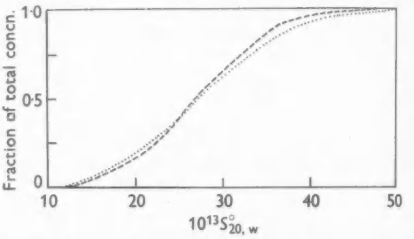

Fig. 3. Sedimentation-coefficient distribution curves at infinite dilution for two preparations of DNA from normal leucocytes: – – –, 20 LD; · · ·, 19 LD.

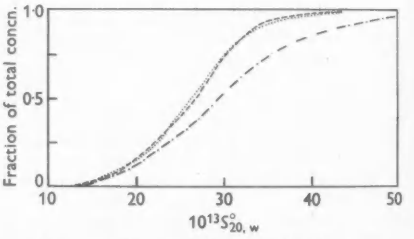

Fig. 4. Sedimentation-coefficient distribution curves at infinite dilution for DNA from spleen: —·—, 3MD (Brill–Simmers disease); · · ·, 4MD (normal); – – –, 5MD (asplastic anaemia).

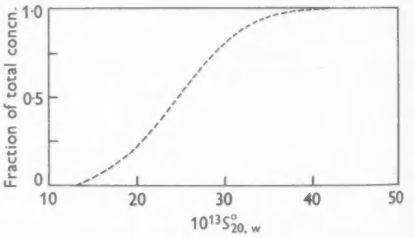

Fig. 5. Sedimentation-coefficient distribution curve at infinite dilution for DNA of the leucocytes from tuberculous empyema, 21 LD.

Table 1. *Comparison of the weight average sedimentation coefficients at infinite dilution and the quartile deviation of the distributions of the preparations of deoxyribonucleic acid*

Sample	Source	Average sedimentation coefficient (s)	Quartile deviation (s)
19LD	Leucocytes, normal	27·0	12·1
20LD		26·8	9·6
2	Leucocytes, lymphatic leukaemia*	18·0	4·6
4		19·4	5·6
6		20·6	5·2
1	Leucocytes, lymphatic leukaemia	30·8	12·4
3		29·5	12·1
5		28·4	12·0
10	Leucocytes, myeloid leukaemia	27·0	12·4
7		24·5	11·6
8		22·8	11·0
9		22·8	8·6
4MD	Spleen, normal	26·2	7·8
5MD	Spleen, aplastic anaemia	26·8	8·2
3MD	Spleen, Brill–Simmers disease	29·8	11·6
21LD	Leucocytes from tuberculous empyema	24·6	8·4

* Prepared by the method of Gulland *et al.* (1947).

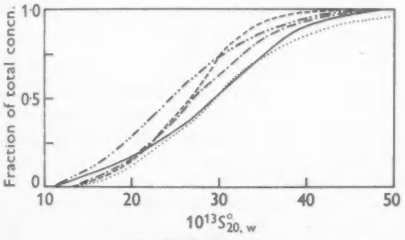

Fig. 6. Comparison of the arithmetic average distribution curves for DNA from the different sources: —·—, normal leucocytes; – – –, non-leukaemic spleen; ···, spleen (Brill–Simmers disease); —, lymphatic leukaemia leucocytes; —··—, myeloid leukaemia leucocytes.

deoxyribonucleic acid from leucocytes of myeloid leukaemia which have been investigated were obtained from three patients. Preparations 7 and 10 were obtained from the leucocytes of one patient, 10 before treatment and 7 at a later stage after treatment with Myleran (busulphan). Myleran therapy (Wintrobe, 1956*b*) induces marked changes in the blood picture. The proportion of immature cells decreases and the number of polymorphonuclear leucocytes increases. The four samples of deoxyribonucleic acid were therefore obtained from cell populations differing both in composition and maturity. The observed changes in the sedimentation characteristics of the deoxyribonucleic acid preparations from leucocytes of myeloid leukaemia might be due to (*a*) variations associated with the average degree of maturity of the cells, (*b*) variations due to the presence of different proportions of the types of cells or (*c*) the occurrence of specific changes of the deoxyribonucleic acid of all the cells. If the last hypothesis is correct it would require the additional postulate that Myleran modifies the physicochemical characteristics of the deoxyribonucleic acid of some of the leucocytes. On the basis of the present results it is not possible to distinguish between these three possibilities. The implications of the first two hypotheses are of some interest. If the properties of the deoxyribonucleic acid of a cell varied with the degree of maturity the changes must be induced by metabolic processes occurring within the cell. If the average characteristics of deoxyribonucleic acid preparations vary with the proportions of different white cells then the characteristics of the deoxyribonucleic acid in the different types of cells cannot be the same. Were either of these hypotheses correct problems of considerable genetic importance would be raised. The reproducibility of the deoxyribonucleic acid preparations from normal leucocytes and from leucocytes of lymphatic leukaemia would appear to be, in each case, a reflexion of the uniformity of the cell population. The cell populations of normal and leukaemic blood are not the same. The differences observed in the sedimentation characteristics of the deoxyribonucleic acid from these two sources may therefore be explained in terms of either of the hypotheses (*b*) and (*c*) above. To distinguish between these two possibilities the properties of the deoxyribonucleic acid of the different types of leucocytes will have to be studied.

If the distribution curves of sedimentation coefficients for deoxyribonucleic acid from normal leucocytes and non-leukaemic spleen in Fig. 6 are compared it will be seen that the lower halves of the curves, from 10 to 27 s, are nearly the same but that the curves differ considerably in the range 27–50 s. In view of the reproducibility observed for the two preparations from each of these sources, the results indicate that the heterogeneity of deoxyribonucleic acid in leucocytes and in spleen is not the same. It may be noted that Bendich, Pahl & Beiser (1956) have demonstrated by chromatography experiments that deoxyribonucleic acid preparations from rat brain and kidney are different. These results suggest that deoxyribonucleic acid may be tissue specific.

Of the three samples of deoxyribonucleic acid from spleen that have been investigated, two (4MD and 5MD) give almost identical distribution curves but the third (3MD, Brill–Simmers disease) is markedly different (Fig. 4). Comparison of the curves of Fig. 6 shows that the distribution observed for 3MD is very similar to that for the deoxyribonucleic acid of leucocytes of lymphatic leukaemia. Brill–Simmers disease frequently develops into lymphatic leukaemia. At the time of the splenectomy histological and clinical investigations did not show the presence of lymphatic leukaemia, but the patient has since developed this disease. The sedimentation results therefore suggest that a change in the nature of the deoxyribonucleic acid to a form which is found in the leucocytes of lymphatic leukaemia has occurred within the spleen at a stage before that at which the disease can be diagnosed by the normal clinical and histological techniques. The implications of this in relation to the development of the disease are being further studied.

It would be of considerable value to determine whether the differences observed between the distribution curves of the various samples studied are due to variations of the molecular weight or the shape of the molecules. It has been shown that the degradation of deoxyribonucleic acid by deoxyribonuclease results in a decrease in molecular weight of the particles without any significant change in shape (Thomas, 1956). Sedimentation experiments (Shooter, 1958) have shown that the action of this enzyme on deoxyribonucleic acid results in a progressive movement of the distribution curve to lower sedimentation coefficients, but the shape of the curve (i.e. the ratio of the weight average sedimentation coefficient to the quartile deviation) is not altered. The shapes of the distribution curves of the samples investigated do not vary very greatly, suggesting that the samples differ in respect of the molecular weight of the particles rather than in their shape. With the deoxyribonucleic acid from the leucocytes of tuberculous empyema (Figs. 5, 6) it is known that the leucocytes undergo nucleolysis and other degradative processes within the pleural cavity.

Investigations of preparations of deoxyribonucleic acid from a variety of sources (Butler & Shooter, 1958) has shown that the quartile deviations of the distributions of sedimentation constants for mammalian deoxyribonucleic acid (calf-thymus, rat-liver) are in the range 12–13 s units, whereas simpler organisms (*Haemophilus influenzae*, T2 and D4 bacteriophage) have quartile deviations in the range 6–10 s units. Of the present series of samples (Table 1), nine have quartile deviations in the range 10–13 s units. Of the exceptions, 2, 4 and 6 may be disregarded since they have been subjected to considerable fractionation during preparation, 21LD is a degraded form of a normal deoxyribonucleic acid and 9 may also be a degraded deoxyribonucleic acid. The two spleen samples (4MD and 5MD) appear to be quite different from other preparations from mammalian sources. This difference is presumably correlated in some way with the function of the spleen. The anomaly of the spleen samples is being further investigated.

SUMMARY

1. Sedimentation-coefficient distribution curves at infinite dilution have been determined for samples of deoxyribonucleic acid from human leucocytes and spleen.

2. The distributions for preparations from normal leucocytes and non-leukaemic spleen indicate that deoxyribonucleic acid exhibits tissue specificity.

3. Differences exist between the distributions observed for deoxyribonucleic acid from normal leucocytes and from leucocytes of myeloid and lymphatic leukaemia.

4. In Brill–Simmers disease changes in the deoxyribonucleic acid of the spleen to a form which is found in the leucocytes in lymphatic leukaemia can occur before the stage at which lymphatic leukaemia can be diagnosed clinically.

5. The deoxyribonucleic acid of leucocytes from tuberculous empyema appears to be a randomly degraded form of that occurring in normal leucocytes.

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The Separation of Flavins by Paper Electrophoresis and its Application to the Examination of the Flavin Contents of Micro-organisms

By J. L. PEEL

Agricultural Research Council Unit for Microbiology, The University, Sheffield 10

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Extracts of *Clostridium kluyveri* are a deep green–brown, but under hydrogen this colour disappears leaving a faint pink colour (Peel & Barker, 1956). Substantially the same observations hold for the rumen organism LC1 of Elsden, Volcani, Gilchrist & Lewis (1956). It appeared likely that the pigments concerned were flavin derivatives, and the presence of such apparently large amounts in these two strict anaerobes was of particular interest. Since very little data were available on the occurrence of flavins in micro-organisms a survey of a variety of species seemed a worthwhile project.

At the outset, suitable analytical methods were not available. It was considered essential that the principal flavin derivatives should be separated from each other in sufficient quantities for confirmatory tests to be applied and in such a manner as to permit an estimate of the amount of each flavin present in the organisms examined. A separation on the required scale has been accomplished by paper electrophoresis and this separation has been combined with a preliminary extraction and concentration procedure adapted from the methods used by Dimant, Sanadi & Huennekens (1952) in the preparation of flavinadenine dinucleotide. This paper describes the method and the results obtained, preliminary accounts of which have already been given (Peel, 1954, 1955).

The electrophoretic separation of flavins has since been reported from a number of laboratories (Siliprandi, Siliprandi & Lis, 1954; Cerletti & Siliprandi, 1955; Gaudiano & Cingolani, 1954; Yagi & Matsuoka, 1955; Walaas & Walaas, 1956).

MATERIALS

Flavin derivatives. Riboflavin was obtained from L. Light and Co. (Colnbrook, Bucks); flavin mononucleotide (FMN) was a gift from Sigma Chemical Co.; riboflavinyl glucoside and one sample of flavinadenine dinucleotide (FAD) were gifts from Dr L. G. Whitby. A crude mixture of FMN and FAD was obtained from baker's yeast by following the procedure of Dimant *et al.* (1952) to the stage before partition chromatography. These products were used without further treatment as markers for paper chromatograms. For recovery experiments riboflavin, FMN and FAD were purified by paper electrophoresis as described later. Lumiflavin was prepared from riboflavin as described by Warburg & Christian (1938). Lumichrome (Karrer, Salomon, Schöpp, Schlittler & Fritzsche, 1934) was prepared as follows: a saturated solution of riboflavin in 0·01 M-ammonium formate–formic acid buffer, pH 4·6, was kept for about 7 days in a shallow layer in daylight at room temperature; HCl was then added to a final concentration of about 0·5 N and the mixture extracted with a half volume of $CHCl_3$. The extract was evaporated to dryness and the residue dissolved in water for use.

Micro-organisms. Sources of micro-organisms and details of the growth conditions and media employed in obtaining cells for the examination of flavin contents are given in Table 7. In experiments on the extraction procedure the large quantities of the rumen organism LC1 required were obtained as described by Walker (1958).

Hexanoate-oxidizing organisms. The three organisms used were isolated from soil by the enrichment-culture technique. The medium contained 1% (w/v) of sodium hexanoate, 0·1 mg. of riboflavin/100 ml. and the following salts (mg./100 ml.): KH_2PO_4, 136; Na_2HPO_4, 213; $(NH_4)_2SO_4$, 50; $MgSO_4,7H_2O$, 20; $CaCl_2,2H_2O$, 0·1; $FeSO_4,7H_2O$, 0·5; $MnSO_4,5H_2O$, 0·25; $Na_2MoO_4,2H_2O$, 0·25; pH 7. Volumes (10 ml.) of medium in 100 ml. Erlenmeyer flasks were inoculated with approx. 2 g. of soil and incubated at 25° or 37°. After 3 days' incubation, the cultures were plated on the above medium solidified by the addition of 2% of washed agar and then incubated at the same temperature as the parent enrichment. After 4 days two types of colony could be distinguished on plates from the culture maintained at 25°. Each type was re-plated twice in succession to obtain pure cultures. The organisms were identified as strains of *Pseudomonas fluorescens* and designated strains CO1 and CO2. The two strains differ in colonial morphology; in addition CO2 grows more slowly than CO1 on all media tested.

Plates from the 37° enrichment showed no growth after 4 days but colonies appeared after 6 days. Only one type of colony was found; this organism was obtained pure by two further platings and identified as *Bacillus sphaericus* var. *rotans*. It was designated strain CO8.

The organisms were maintained on slopes of the medium given in Table 7 with 0·1% of sodium hexanoate as carbon source and solidified with 2% of washed agar.

METHODS

Separation and estimation of flavins

Extraction and concentration of flavins. Micro-organisms (approx. 2 g. dry wt.) were harvested from cultures with the Sharples Super-centrifuge and washed once by resuspending in 100–200 ml. of water and recentrifuging in tared tubes. The weight of cell paste was determined and the cells were resuspended in water to give approx. 200 mg. of wet cells/ml. Duplicate samples of 1 ml. were removed and dried at 110° for 4 hr. to determine the dry weight of organism present. To the remainder of the suspension was added 0·25 vol. of 50% (w/v) trichloroacetic acid, the whole mixed thoroughly and allowed to stand for 5 min. and the insoluble material removed by centrifuging at 9000 *g* for 10 min. In all subsequent operations reagents were chilled to 2° before use and, where possible, vessels were immersed in ice–water. Exposure to light was minimized by working in the dark or in artificial light.

The flavins were next removed from the trichloroacetic acid extract by adsorption on a column of Florisil. The column was of 11 mm. bore and contained 6 g. (12 ml.) of 30/60-mesh Florisil (Floridin Co., Tallahassee, Florida, U.S.A.) bounded at top and bottom by a plug of glass-wool. The Florisil was soaked in 0·1 N-HCl for at least 16 hr. before making up the column, after which it was washed with water until the pH of the effluent rose to between 6 and 7. A known volume of trichloroacetic acid extract containing 0·2–1·0 μmole of total flavin was run on to the column as rapidly as possible and washed in with a few millilitres of 2% (v/v) acetic acid. The flow rate was then reduced to 5–10 ml./min. and the column washed successively with 100 ml. of 2% (v/v) acetic acid, 100 ml. of water and 100 ml. of aq. 0·5% (v/v) pyridine. During the first two washes the flavins remained adsorbed as a yellow band at the top of the column, but moved slowly downwards on application of the 0·5% pyridine. When necessary this last washing was curtailed if the flavins approached the bottom of the column before the whole 100 ml. had been applied.

The flavins were next displaced from the column with aq. 25% (v/v) pyridine. Normally the yellow flavin fraction came off the column in 10–15 ml. In some cases where other pigments obscured the end of the flavin band, 20 ml. was collected after the flavin first began to emerge. Pyridine was removed from the flavin fraction by three extractions with 5–10 vol. of $CHCl_3$ followed by one extraction with ether. Dissolved ether was removed by aeration and the pH adjusted to 7 with NaOH. The volume and colour intensity of the resulting solution were then measured (stage A), after which the solution was freeze-dried. The solid product was extracted with a known volume of water, usually 1–2 ml., centrifuged and the supernatant used for paper electrophoresis.

A small precipitate was often observed on centrifuging (cf. Dimant *et al.* 1952), the amount varying with the sample of Florisil. It was reduced to 2–3 mg. dry wt. by selecting a suitable batch of adsorbent, by pretreating with HCl and by using 25% pyridine as eluent, which brings the flavins off the column in a smaller volume than the 5% pyridine used by Dimant *et al.* Under these conditions the amount of flavin remaining adsorbed on the precipitate was negligible.

Separation of flavins by paper electrophoresis. The apparatus used was that of Markham & Smith (1952). The flavin concentrate was applied to the paper strip (Whatman no. 3MM, 60 cm. × 10 cm.) as a transverse streak 8 cm. long and approx. 8 cm. from the middle of the paper. Between 0·1 and 0·2 μmole of total flavin was applied. In drying the solutions applied to the paper the use of hot air was avoided. The paper was next immersed in buffer except for a transverse strip 1 cm. wide containing the flavin streak, which was allowed to moisten by capillarity. Surplus buffer was removed from those parts of the paper which had been immersed by blotting between clean filter paper. The buffer was 0·025 M-formic acid–ammonium formate, pH 4·6, and was replaced after each experiment. Higher buffer concentrations resulted in a higher current and hence increased heat output, whereas lower concentrations did not maintain a constant pH along the paper strip throughout electrophoresis. This was tested by spotting drops of indicator along the paper after the experiment.

Electrophoresis was for 2 hr., with an initial potential difference of 1000 v between the electrodes, giving a potential gradient of approx. 20 v/cm. and a current of 10 mA. During the experiment the current rose to 20 mA. the potential difference fell to 900 v and the temperature of the CCl_4 used as coolant rose about 20° above room temperature. The coolant was either replaced between experiments or allowed to cool and dried with pieces of filter paper.

After electrophoresis, the paper was dried at room temperature and the flavins were located by their yellow colour and yellow fluorescence under ultraviolet light. When a permanent record was required, the paper was photographed with panchromatic film and a 3 × yellow filter (Actina, London). The distance from camera lens to object was 3 ft., the lens aperture *f* 3·5, the film Ilford FP3 and the illumination was provided by a strip ultraviolet lamp (Hanovia Ltd., Slough, Bucks) placed 2 ft. from the

object. Under these conditions an exposure of 60 sec. was satisfactory. Legends were written on strips of paper with a saturated solution of 5-aminoacridine as a fluorescent ink.

The mobilities of some flavin derivatives are given in Table 1. Riboflavin, FMN, FAD and lumichrome are readily separated but the conditions do not permit the adequate separation of riboflavin from lumiflavin and riboflavinyl glucoside. This limitation was not important for the present purpose.

After location of the flavin bands, strips containing the individual components were cut out and eluted with water (Dent, 1947) until the eluate was colourless and no fluorescence could be detected on the paper under ultraviolet light: about 0·5 ml. of eluate was required/cm. width of the strip. The volumes and colour intensities of the eluates were measured (stage B), after which they were concentrated by freeze-drying, where necessary, before being used for confirmatory tests.

Recoveries. When a mixture of riboflavin, FMN and FAD was treated with trichloroacetic acid and then subjected to the above procedure, the recovery of total flavin up to stage A was about 80% (Table 2). No loss of flavin was observed between stage A and the extraction of the freeze-dried material. In the routine procedure, the flavins were exposed to trichloroacetic acid for a total time of about 20 min., 5 min. being allowed for manipulations. Electrophoretic analysis showed that, with this treatment, the composition of the flavin mixture after freeze-drying differed slightly from that of the starting material; the proportion of FAD decreased. The decrease was much more marked after 60 min. exposure to trichloroacetic acid and was due mainly to a breakdown of FAD to FMN (cf. Bessey, Lowry & Love, 1949). Recoveries over the electrophoretic separation were approx. 90% for riboflavin, FMN and FAD, singly or in combination (Table 3).

Spectrophotometric measurements. Flavin solutions were assayed by measuring the intensity of their yellow colour. For standardizing flavin solutions, and in some preliminary experiments, the Unicam SP. 500 instrument was used and the amount of flavin calculated from the optical density at 450 mμ, with the values for molecular-extinction coefficients (ϵ) given by Whitby (1953), i.e. $12{\cdot}2 \times 10^3$ l. mole^{-1} cm.$^{-1}$ for riboflavin and FMN; $11{\cdot}3 \times 10^3$ l. mole^{-1} cm.$^{-1}$ for FAD. In routine experiments, extinctions were measured in the Hilger-Spekker absorptiometer with the Ilford no. 601 filter (maximum transmission at 430 mμ, transmission at 450 mμ about half of the maximum) and with microcells of 0·5 ml. capacity and 1 cm. light path. This instrument was calibrated separately for riboflavin, FMN and FAD with electrophoretically purified samples of each, standardized with the Unicam SP. 500 instrument.

With the Spekker instrument, the experimentally determined values of ϵ were identical for riboflavin and FMN ($\epsilon_{RBF} = \epsilon_{FMN} = 11{\cdot}0 \times 10^3$ l. mole^{-1} cm.$^{-1}$) but the value for FAD was somethat lower ($\epsilon_{FAD} = 9{\cdot}8 \times 10^3$ l. mole^{-1} cm.$^{-1}$). For mixtures of these three flavins, the appropriate molecular extinction coefficient (ϵ_M) could be obtained, provided that the molar proportion of FAD (x) was known, from the equation:

$$\epsilon_M = (1 - x)\,\epsilon_{RBF} + x.\epsilon_{FAD}.$$

This relationship was therefore used where extinction measurements on mixtures were used to determine total flavin. Calculation of flavin contents of micro-organisms from extinction measurements is described later under Results.

Table 1. *Electrophoretic mobilities of some flavins*

Potential gradient 20 v/cm.; duration of experiment 2 hr. Buffer 0·025 M-formic acid–ammonium formate, pH 4·6. Other details are given in the text.

Substance	Distance travelled towards anode (cm.)
Riboflavin	−1·6
FMN	10·1
FAD	15·0
Lumiflavin	−1·3
Riboflavinyl glucoside	−1·8
Lumichrome	−0·2

Confirmatory tests on separated flavin fractions

No tests were done with the riboflavin fractions as these usually contained insufficient flavin. Tests on the FMN and FAD fractions were carried out as follows.

Table 2. *Recovery of flavins during treatment before electrophoresis*

To flavins contained in 20 ml. (Expt. 1) or 13 ml. (Expt. 2) at room temp. (19°), 0·25 vol. of 50% trichloroacetic acid was added and the mixture was applied to the Florisil column after the time indicated. Procedure described in the text was then followed and the composition of the initial and final flavins was determined by electrophoresis. Total flavin was calculated from the extinction at each stage as indicated in the text. In calculating the total flavin at stage A, the proportions of the three flavins were assumed to be the same as after freeze-drying.

	Time of standing with trichloroacetic acid (min.)	Stage of treatment	Total flavin (μm-moles)	Recovery (%)	Flavin in each fraction (%)		
					Riboflavin	FMN	FAD
Expt. 1	20	Initial flavin	597	—	9	41	50
		Before freeze-drying (stage A)	462	78*	—	—	—
		Extract of freeze-dried material	—	—	11	45	44
Expt. 2	60	Initial flavin	889	—	9	41	50
		Before freeze-drying (stage A)	728	82*	—	—	—
		Extract of freeze-dried material	731	100†	11	52	37

* As percentage of initial flavin.

† As percentage of flavin at stage A.

Table 3. *Recovery of flavins during electrophoresis*

Details of electrophoresis and assay of solutions are given in the text. Initial proportions of the three flavin compounds in the mixture were unknown, but were assumed to be the same as for the recovered flavins in calculating the initial total flavin as indicated in the text.

Test substance	Initial flavin (μm-moles)	Recovered flavin (μm-moles)	Recovery (%)
Single flavins			
Riboflavin	53	49	93
FMN	59	54	91
FAD	67	61	92
Mixture			
Riboflavin	—	21	—
FMN	—	49	—
FAD	—	92	—
Total	181	162	90

Electrophoresis and chromatography on paper. Electrophoresis was carried out under the same conditions as the original separation but the flavins were applied as spots, and markers were also run on the same strip. The four solvents used for chromatography were: 1, aq. 5% Na_2HPO_4, single phase, ascending (Carter, 1950); 2, water saturated with *iso*pentanol, single phase, ascending (Whitby, 1952); 3, acetone–*iso*butyric acid–water (55:15:30 by vol.), single phase, ascending; 4, phenol–butan-1-ol–water (160 g.:30 ml.:100 ml.) lower phase mobile, descending (Dimant *et al.* 1952). Whatman no. 1 filter paper was used throughout and flavins were applied and located as in electrophoresis. Phenol quenches the fluorescence of flavins and had to be removed after using solvent 4. The chromatogram was therefore dried at room temperature, and then washed twice with xylene and once with ether. Riboflavin, FMN and FAD markers were included on all chromatograms.

D-*Amino acid oxidase test.* Tests for FAD were done with the apoenzyme of D-amino acid oxidase as described by Warburg & Christian (1938). Samples of eluates containing 3–10 μm-moles of flavin were used. The same quantities of FAD increased the uptake of O_2 by 100–300 μl./hr.

Behaviour of other flavins. During recent years, a number of new flavin derivatives have been obtained from biological materials and their behaviour in the present procedure must be considered. Riboflavinyl glucoside (Whitby, 1952) has almost the same electrophoretic mobility as riboflavin and would not be distinguished from it, since no confirmatory tests were carried out on the riboflavin fraction. To date it has not been reported in bacteria though Tachibana & Katagiri (1955*a*) found it in the medium after growth of a mutant of *Aspergillus oryzae*. The electrophoretic behaviour of the FAD-X of Huennekens, Sanadi, Dimant & Schepartz (1953), the 'fourth flavin compound' of Yagi (1953) and the riboflavin diphosphate of Tachibana & Katagiri (1955*b*) is unkown. However, FAD-X is known to be inactive in the D-amino acid oxidase test and separates from riboflavin, FMN and FAD in solvent 4 (Dimant *et al.* 1952). Yagi's compound separates in solvent 1 (K. Yagi, personal communication). The behaviour of riboflavin diphosphate in the confirmatory tests is unknown but in view of its chemical nature the possibility that it would remain undetected in all the tests employed seems remote.

The presence of lyxoflavin in human heart has been reported by Pallares & Garza (1949). This compound differs from riboflavin solely in the configuration of C-4 of the pentose residue and cannot at present be distinguished from riboflavin on a small scale. In what follows, it has been assumed for convenience that the flavins found in micro-organisms are all derivatives of riboflavin, but the possible occurrence of the corresponding lyxoflavin derivatives is not excluded.

Choice of extraction procedure

Extracting agent. Methanol (Kuhn, Wagner-Jauregg & Kaltschmidt, 1934), acetone (Euler & Adler, 1934), Takadiastase (Emmett, Bird, Brown, Pescock & Vandenbelt, 1941), trichloroacetic acid (Bessey *et al.* 1949; Dimant *et al.* 1952) and various forms of heat treatment (Warburg & Christian, 1938; Hodson & Norris, 1939; Yagi, 1951; DeLuca, Weber & Kaplan, 1956) have all been used for the extraction of flavins from biological materials. However, only Yagi (1951) and Bessey *et al.* (1949) have been concerned with the separate estimation of riboflavin, FMN and FAD and have examined the fate of these compounds when subjected to their analytical procedures. These authors worked almost exclusively with animal tissues. Bessey *et al.* extracted homogenized tissues with 10% trichloroacetic acid at 0° for 15 min.; Yagi heated for 2 min. at 80°, homogenized and then heated again at 80° for a further 15 min. Although trichloroacetic acid causes a slow breakdown of FAD at the temperature employed here, it was preferred in the present work because (*a*) heat treatment may produce artifacts under some conditions: thus Yagi (1953) found that if in his procedure the second heat treatment were modified by heating at 90° for 1 hr. a new flavin was obtained from liver, and the same material could be obtained by heating FAD alone at 95°; (*b*) heating procedures do not completely remove protein, necessitating further treatment (cf. Warburg & Christian, 1938; Yagi, 1951). Thus extracts of dried cells of the rumen organism LC1 prepared according to DeLuca *et al.* (1956) are green; on adding trichloroacetic acid to a final concentration of 10% a heavy white precipitate is formed and the colour of the supernatant changes to the characteristic yellow of free flavins.

Extraction conditions. Optimum conditions for extraction of flavins by trichloroacetic acid were established for the rumen organism LC1. This organism has a relatively high flavin content and the yellow colour of extracts is probably entirely due to flavin, since (*a*) no other chromogens were detected in any of the fractions when the flavins of this organism were examined as described above; (*b*) extracts prepared by various methods are almost completely bleached on adding sodium dithionite, and extracts prepared from dried cells according to Stadtman & Barker (1949) are bleached by H_2. It was therefore possible to use the colour intensity to follow the extraction of flavins from this organism by trichloroacetic acid.

From the results (Table 4) extraction with 10% trichloroacetic acid for 5 min. at room temperature was selected as the most suitable procedure. These conditions ensure maximum extraction and avoid the slow fall in flavin content of the extract which occurs after longer treatment. No advantage was gained by increasing the

concentration of trichloroacetic acid and at a lower temperature (10°) the extraction was less rapid initially.

Efficiency of extraction. The above experiments establish optimum conditions for extraction with trichloroacetic acid but do not imply that this agent is completely effective either in rendering the cells permeable or in dissociating flavins from flavoproteins. Trichloroacetic acid appears to destroy the permeability barriers of the microbial cell since, when cells of LC1, yeast or *Desulphovibrio* were pretreated either by acetone-drying or by crushing in a bacterial press, no increase was observed in the total extractable flavin (Table 5). There is therefore no advantage in such pretreatments; in fact it is better to omit them since both the crushing of LC1 and the acetone-drying of *Desulphovibrio* lead to a decrease in extractable flavin. In addition, whereas these treatments are without effect upon the relative amounts of riboflavin, FMN and FAD extracted from yeast and LC1 (Table 5), acetone-drying of *Desulphovibrio* results in a marked breakdown of FAD to FMN. The analysis of acetone-dried powders in lieu of fresh cells may therefore give erroneous results.

As regards the effectiveness of trichloroacetic acid in dissociating flavins from protein, it was observed with the organism LC1 that whereas whole cells are a deep green–brown, the residue after extraction with the acid is almost pure white, showing that the release of flavin is virtually complete. In applying the method it has been assumed that this holds true for other organisms. In some cases the residues after extraction were not colourless, e.g. with *Micrococcus lysodeikticus* a bright-yellow residue of a hue different from that of flavoproteins was obtained. In such cases the residue was resuspended in water, heated for

Table 4. *Extraction of flavin from cells of LC1 with trichloroacetic acid*

Sufficient trichloroacetic acid to give the required final concentration was added in a volume of 3·3 ml. to samples (6·7 ml.) of a suspension of washed cells of the organism LC1 (approx. 375 mg. of wet cell paste/ml.). After the times indicated, the mixtures were centrifuged for 5 min. at 9000 *g* and the optical density of the supernatant was determined with the Hilger-Spekker instrument. Trichloroacetic acid (25%) has zero optical density under the conditions used.

	Concn. of trichloroacetic acid (%)	Temp.	Optical density 0 min.	1 min.	5 min.	10 min.	30 min.	60 min.
Expt. 1	2	20°	0·16	—	—	0·15	0·19	0·24
	4	20	0·19	—	—	0·39	0·60	0·58
	8	20	0·58	—	—	0·77	0·67	0·66
	16	20	0·80	—	—	0·79	0·72	0·74
Expt. 2	10	20	—	—	0·62	—	—	—
	15	20	—	—	0·60	—	—	—
	20	20	—	—	0·59	—	—	—
	25	20	—	—	0·63	—	—	—
Expt. 3	10	20	—	0·76	—	0·70	0·69	0·66
	10	10	—	0·63	—	0·71	0·68	0·67

Table 5. *Effect of acetone-drying and crushing of cells on extractable flavins*

A different preparation of washed cell paste (or pressed cake of baker's yeast) was used in each experiment. Where indicated, one-half of the preparation was crushed without abrasive in a bacterial press (Hughes, 1951) or acetone-dried according to Umbreit, Burris & Stauffer (1949); in the latter case, the precipitated material was washed with acetone then ether before drying. Flavins were extracted as described in the text. With the acetone-dried powder a suspension containing 85 mg. dry wt./ml. was used and with crushed cells each millilitre of suspension contained the product from 200 mg. of wet cells. Flavin contents are given in terms of dry wt. of the original cells. Values in parentheses are in terms of dry wt. of acetone-dried powder.

Expt.	Organism	Pretreatment	Total flavin (μm-moles/g. dry wt.)	Flavin in each fraction (%) Riboflavin	FMN	FAD	Calculation method for total flavin (see text)
1	LC1	None	1330	0	31	69	2
		Acetone-dried	1300	0	29	71	2
2	LC1	None	1470	2	27	71	2
		Crushed	1050	2	27	71	2
3	Baker's yeast	None	—	3	54	43	2
		Acetone-dried	—	1	49	50	2
4	Baker's yeast	None	65	5	44	51	2
		Crushed	63	6	42	52	2
5	*Desulphovibrio*	None	116	0	21	79	1
		Acetone-dried and stored for 4 days at room temp.	61 (105)	0	44	56	1
6	*Desulphovibrio*	Acetone-dried and stored for 20 hr. at room temp.	— (122)	1	63	36	1

20 min. at 100° and recentrifuged. No change was ever apparent in the colour of the residue, though in a few cases a faint-brown colour appeared in the supernatant.

It thus seems probable that the present procedure extracts virtually all the flavin of microbial cells. However, recent studies by Boukine (1955) and by Kearney & Singer (1955) with the succinic dehydrogenase of animal tissues have demonstrated that the flavin component is not completely released by boiling or treatment with cold acid. On treating with proteolytic enzymes, a flavin derivative is released which probably contains FAD linked to a peptide (Singer, Kearney & Massey, 1956). Such derivatives would not be extracted by the procedure usually employed for the extraction of flavins. In assessing the present results it should therefore be remembered that they refer to flavin extractable with trichloroacetic acid.

RESULTS

Qualitative aspects. Electrophoretic fractions in the positions corresponding to FMN and FAD were obtained from all the organisms examined. With most organisms a faint band was also obtained in the riboflavin position. These were the only fractions which were yellow in visible light and fluoresced yellow under ultraviolet light and were therefore presumably the only derivatives of riboflavin. In some cases a faint band was observed which fluoresced pale blue under ultraviolet light and which remained on or near the starting line. This was presumed to be lumichrome formed by the breakdown of some of the flavin during manipulation.

With several organisms other pigments were observed. When the extract of *Desulphovibrio* with trichloroacetic acid was applied to the Florisil column, the band which formed at the top was deep green instead of the usual yellow. During the wash with 0·5% pyridine, a blue pigment was eluted which turned red on adding excess of alkali and was presumably the prosthetic group of the 'desulphoviridin' of Postgate (1956). *Propionibacterium pentosaceum*, *M. lysodeikticus*, *Pseudomonas* OD 1, *Rhodospirillum rubrum* and *Chlorobium thiosulphatophilum* gave rise to a red–brown band on the Florisil. Much of the contaminating pigment remained in the flavin fraction after elution but separated upon electrophoresis to give a red-brown band, which fluoresced faintly blue under ultraviolet light and migrated towards the cathode with a mobility about double that of riboflavin. A different material having a similar mobility was observed in preliminary experiments with baker's yeast. This material fluoresced orange under ultraviolet light but was not detectable in visible light. It was not observed in all batches of baker's yeast and was not present in the pure culture of *Saccharomyces cerevisiae* grown as described in Table 7.

The identity of the FMN and FAD fractions was confirmed in all cases by the tests described above. The results with LC 1 are given in Table 6 as an example. In the chromatographic tests, traces of substances other than the main component were often observed. These were always in positions corresponding to known decomposition products, e.g. traces of FMN and lumichrome were detected in some FAD fractions and were attributed to decomposition during the manipulations after electrophoresis.

Calculation of quantitative results. The recovery experiments (Tables 2 and 3) show that when the procedure adopted for extraction and separation of flavins is applied to known mixtures of riboflavin,

Table 6. *Confirmatory tests on the flavin mononucleotide and flavinadenine dinucleotide fractions of the rumen organism LC 1*

Experimental details are given in the text. Rates of oxygen uptake were measured over the period 0–60 min.

1. Chromatography and electrophoresis

	Chromatography R_F values				Electrophoresis distance moved towards anode (cm.)
	Solvent 1	Solvent 2	Solvent 3	Solvent 4	
Markers					
Riboflavin	0·27	0·38	0·52	0·79	−1·1
FMN	0·49	0·90	0·26	0·09	8·6
FAD	0·35	0·89	0·07	0·15	14·2
Fractions from LC1					
FMN	0·50	0·89	0·26	0·10	8·8
FAD	0·35	0·89	0·07	0·16	14·3

2. D-Amino acid oxidase test

Test substance	Rate of O_2 uptake (μl./hr.)
None	−5
Authentic FAD (8 μm-moles)	138
Fractions from LC1	
FMN (7 μm-moles)	10
FAD (8 μm-moles)	140

FMN and FAD, substantial losses of flavin occur, although the relative proportions of the three flavins change only slightly. Consequently, in order to obtain a value for the flavin content of the original cells, correction factors have to be introduced and certain assumptions become necessary. The assumptions which have been made are: (*a*) that the proportions of the three flavins do not change during the manipulations; (*b*) that after extraction with trichloroacetic acid the flavins are evenly distributed throughout the total volume of cells plus extractant; (*c*) that the recovery of flavins up to stage A is 80 %; (*d*) that the recovery between stages A and B is 90 %. This last assumption is only necessary when interfering pigments are present at stage A.

The amounts of flavin in the individual fractions after electrophoresis were obtained from the measurements at that stage (B). The relative proportions of the three flavins at stage B could thus be found and by assumption (*a*) this gave the relative proportions in the original cells and at any stage of the manipulations.

The total flavin content of the original cells (F) was calculated from the total flavin at either stage A or stage B (F_A, F_B respectively) according as interfering pigments were absent or present at stage A, the following relationships being used:

$$F = F_B \cdot \frac{100}{80} \cdot \frac{100}{90} \cdot \frac{V_E}{mV_c} \quad \text{Method 1}$$

$$F = F_A \cdot \frac{100}{80} \cdot \frac{V_E}{mV_c} \quad \text{Method 2}$$

where V_E = total volume of cells plus extractant; V_c = volume of extract put on to Florisil column; m = dry wt. of cells extracted.

With Method 1, F_B was obtained by summing the amounts of the individual flavins at stage B. With Method 2, the measurements on the mixture at stage A were used to calculate F_A, with the appropriate value for the molecular extinction coefficient (ϵ_M) obtained as described in the Methods section. For this purpose it was necessary to know the molar proportion of FAD (x), and this was obtained from the measurements at stage B with assumption (*a*). In a few cases where quantitative data on the three individual flavin fractions were not obtained at stage B, it was assumed that $x = 0{\cdot}5$. The maximum possible error so introduced in the value for F is 6 %.

Both methods 1 and 2 involve assumptions (*b*) and (*c*). Method 1 involves in addition assumption (*d*) and also necessitates quantitative handling between stages A and B which is inconvenient on account of the small volumes used. Method 2 does not have these disadvantages, and although it brings in assumption (*a*), this is required in any case to obtain the relative proportions of the three flavins in the original cells. In calculating F therefore method 2 is preferable but can be used only when no interfering pigments are present at stage A. Furthermore, it is possible to dispense with the quantitative handling between stages A and B only if it is known before electrophoresis that no contaminating pigments are present. Fortunately, it was possible with a little experience to decide if this were so from the appearance of the concentrate at stage A and in all cases where such pigments were judged to be absent by this means, none was found on subsequent electrophoresis. Method 2 was therefore used when no contaminating pigments were present, otherwise method 1 was used.

With *Thiobacillus denitrificans*, the value for total flavin could not be obtained as the cells harvested were contaminated with large amounts of sulphur.

Quantitative aspects. With the exception of the hexanoate-oxidizing organisms, which will be considered separately, the micro-organisms examined and results obtained are given in Table 8 in ascending order of flavin content. Details of the organisms and their growth are given in Table 7. There is a wide range of flavin contents, from 30 to 2930 μm-moles/g. dry wt. with *Rhodospirillum rubrum* and *Clostridium kluyveri* respectively. In general, the higher flavin contents were associated with anaerobic growth. For example, anaerobically grown cells of *Saccharomyces cerevisiae* were found to contain about 50 % more flavin than aerobically grown cells, in agreement with the earlier observations of Pett (1935) and of Singh, Agarwal & Peterson (1948). The highest amounts of flavin were found in those strict anaerobes producing, as fermentation products, higher volatile fatty acids (HVFA) containing four or more carbon atoms. These organisms all contained more than 450 μm-moles/g. dry wt., whereas the highest value observed with a non-HVFA-producer was 360 μm-moles/g. dry wt. The HVFA-producers are all members of the genus *Clostridium* with one exception, the rumen organism LC 1, which though morphologically distinct from the members of this genus resembles them biochemically (Elsden *et al.* 1956). Outstandingly high flavin contents were found in LC 1 and *Cl. kluyveri*, which ferment lactate and ethanol respectively to yield mainly HVFA.

The data on the proportion of flavin present in the three fractions show that virtually all the flavin is present in the two known coenzyme forms FMN and FAD. Indeed, except with *Cl. skatologenes* and possibly a few other organisms, the proportion of free riboflavin is so low as to suggest that it might well have arisen by breakdown of FMN

Table 7. *Organisms and growth conditions*

Under 'Source', Sheffield, NCIB and NCTC refer respectively to organisms maintained in the Microbiology Department, Sheffield University, the National Collection of Industrial Bacteria and the National Collection of Type Cultures. Yeast autolysate was prepared according to Barker & Beck (1942). Pronutrin is a dried commercial casein digest (Herts Pharmaceuticals Ltd., Welwyn) now unobtainable. Penicillin flasks (Jayasuriya, 1955) contained 300 ml. of medium and were subjected to continuous mechanical shaking where indicated. For 'deep culture' Erlenmeyer flasks (3–5l capacity) contained medium to within a few inches of the top. The two photosynthetic organisms were grown in full Roux bottles closed with rubber bungs. When gas phases other than air were used, culture vessels were designed to allow gas to be bubbled through the culture initially and to allow gas produced during fermentation to escape through a valve. H_2–CO_2 and N_2–CO_2 mixtures both contained 5% (v/v) of CO_2.

Organism	Source	Medium	Growth conditions: Duration (hr.)	Temp.	Initial gas phase	Other details
1. *Rhodospirillum rubrum* S1	NCIB 8255	Malate–citrate–yeast extract–salts medium of Elsden & Ormerod (1956)	96	25°	None	Full Roux bottles with illumination
2. *Micrococcus lysodeikticus*	NCTC 2665	1% Glucose, 0·5% Pronutrin, 2% (v/v) yeast autolysate, 2% agar. pH 7	72	37	Air	Surface growth in Roux bottles
3. *Saccharomyces cerevisiae*	Sheffield	2% Glucose, 10% (v/v) yeast autolysate. pH 7	22	25	Air	Penicillin flasks with shaking
4. *Desulphovibrio desulphuricans* (Hildenborough)	NCIB 8303	0·35% Sodium lactate, 0·4% Difco yeast extract, 0·2% $MgSO_4,7H_2O$, 0·1% Na_2SO_4, 0·1% NH_4Cl, 0·05% K_2HPO_4, 0·01% $CaCl_2$, 0·1 mM-$Na_2S,9H_2O$. pH 7·5	72	30	H_2–CO_2	—
5. *Azotobacter agile*	NCIB 8004	0·5% Mannitol, 0·02% calcium acetate, 0·1% K_2HPO_4, 0·02% $MgSO_4,7H_2O$, 0·0002% Na_2MoO_4, 0·0001% $FeSO_4,7H_2O$, 0·0001% NaCl. pH 7·6	41	25	Air	Penicillin flasks with shaking
6. *Bacillus subtilis*	NCTC 3610	2% Glucose, 2% (v/v) yeast autolysate. pH 7	18	25	Air	Penicillin flasks with shaking
7. *Saccharomyces cerevisiae*	As no. 3	As no. 3	22	25	Air	Deep culture
8. *Bacillus megaterium*	NCIB 8508	2% Glucose, 2% (v/v) yeast autolysate. pH 7	18	25	Air	Penicillin flasks with shaking
9. *Lactobacillus casei*	NCTC 8010	2% Pronutrin, 2% glucose, 1% sodium acetate ($2H_2O$), 0·45% KH_2PO_4, 4% (v/v) yeast autolysate, 0·5% (v/v) of salts B mixture of Barton-Wright (1946). NaOH to pH 7·6	17	37	Air	Deep culture
10. *Pseudomonas* OD1	Sheffield	Oxalate–salts medium A of Jayasuriya (1955)	48	25	Air	Penicillin flasks

10. *Pseudomonas* OD1	Sheffield	Oxalate–salts medium A of Jayasuriya (1955)	48	25	Air	Penicillin flasks
11. *Escherichia coli* K4	Sheffield	2% Glucose, 5% (v/v) yeast autolysate, 0·1% Marmite, 1% Pronutrin. pH 7	22	31	Air	Penicillin flasks with shaking
12. *Chlorobium thiosulphatophilum*	Dr H. Larsen	Thiosulphate–bicarbonate–salts medium of Larsen (1953)	72	30	None	Full Roux bottles with illumination
13. *Lactobacillus helveticus*	NCTC 4113	Glucose–peptone–growth factors–salts. Riboflavin-free medium of Barton-Wright (1946) with xylose omitted and 10 μg. of riboflavin/100 ml. added	22	37	Air	Deep culture
14. *Propionibacterium pentosaceum* E21	Dr C. B. van Niel	1·4% Sodium lactate, 10% (v/v) yeast autolysate. pH 7	70	30	N_2–CO_2	—
15. *Clostridium welchii* SR12	NCTC 6784	2% Pronutrin, 10% (v/v) supernatant from Robertson's meat medium	6	37	H_2–CO_2	—
16. *Pseudomonas stutzeri*	Dr C. B. van Niel	10% (v/v) Yeast autolysate, 0·5% KNO_3. pH 7	17	30	Air	Deep culture
17. *Pseudomonas aeruginosa*	NCTC 6750	10% (v/v) Yeast autolysate. pH 7	16	25	Air	Penicillin flasks with shaking
18. *Clostridium sporogenes*	Sheffield	1% Glucose, 1% Pronutrin, 0·5% Marmite, 2% (v/v) supernatant from Robertson's meat medium. pH 7	12	37	H_2–CO_2	—
19. *Clostridium skatologenes*	Dr R. F. Rosenberger	1% Glucose, 1% Pronutrin, 3% (v/v) yeast autolysate. pH 7	44	37	H_2–CO_2	—
20. *Clostridium butyricum*	NCIB 6184	2% Glucose, 5% (v/v) yeast autolysate. pH 7	17	37	H_2–CO_2	—
21. Rumen organism LC1	Sheffield NCIB 8927	Lactate–corn-steep liquor medium (Walker, 1958)	16	37	H_2–CO_2	—
22. *Clostridium kluyveri* K1	Dr H. A. Barker	Ethanol–salts medium of Stadtman & Barker (1949) but with biotin plus *p*-aminobenzoic acid replaced by 0·05% of Difco yeast extract	48	37	H_2–CO_2	—
23. *Thiobacillus denitrificans*	Sheffield	Thiosulphate–nitrate–salts medium of Baalsrud & Baalsrud (1952) as modified by Trudinger (1956)	96	28	Air	Deep culture
Hexanoate-oxidizing organisms	Soil isolates					
24. *Pseudomonas* CO1	NCIB 8865	0·2% Na_2HPO_4, 0·1% KH_2PO_4, 0·1% $(NH_4)_2SO_4$, 0·1% (v/v) yeast autolysate, 0·04% $MgSO_4,7H_2O$. Carbon source as indicated in Tables 9 and 10. pH 7	See Table 9	25	Air	Penicillin flasks
25. *Pseudomonas* CO2	NCIB 8866		See Table 9	25	Air	Penicillin flasks
26. *Bacillus sphaericus* var. *rotans* CO8	NCIB 8867		See Table 9	37	Air	Penicillin flasks

and FAD in the steps before electrophoresis. The situation is thus similar to that in animal tissues, where Bessey *et al.* (1949) and Yagi (1951) found that in general free riboflavin accounts for less than 3% of the total flavin in normal tissues. The present values for the proportion of FAD should be regarded as minimum figures since there is a small breakdown of FAD to FMN under the conditions of extraction (Table 2), and this has not been corrected for. Nevertheless, it is clear that micro-organisms differ from animal tissues in the proportion of flavin present as FAD. In a variety of animal tissues most of the flavin is FAD, whereas in micro-organisms proportions of FMN and FAD vary from 30% of FAD and 68% of FMN with *Lactobacillus helveticus* to 79% of FAD and 21% of FMN with *Desulphovibrio*. Within the group of HVFA-producers there is much less variation and the proportion of FAD is high.

The data already in the literature on the flavin contents of micro-organisms are fragmentary. Results calculated from the data of other authors are given in parentheses in Table 8: where necessary it has been assumed that wet cells contain 20% of dry matter. In almost all cases the media and growth conditions have not been adequately described and comparison is therefore difficult, but in general there is rough agreement with the present results. An exception is the value of 20 μm-moles/g. dry wt. for *Bacillus subtilis* found by Pett (1935), which is low compared with the present results for *Bacillus* species. Pett used an early method which was relatively crude, being based upon the comparison of the fluorescence of an ethanolic extract with that of a standard solution of riboflavin. It is noteworthy in this respect that Pett failed to detect flavin in *Escherichia coli*, although he reported that his extract showed a blue fluorescence. The present results are therefore probably more reliable. The value of 480 μm-moles/g. dry wt. for total flavin in *Lactobacillus arabinosus* (DeLuca *et al.* 1956) is much higher than values found here for two lactobacilli, and the figure (96%) of DeLuca *et al.* for the proportion of FAD is much higher. These authors also report a remarkable finding with *Cl. kluyveri*. The total flavin content of the organism when grown on synthetic medium (1830 μm-moles/g. dry wt.) was comparable with the present result, but when the organism was grown on 'complex medium', the flavin content was about 8% of this value. The significance of this result is difficult to assess as the authors give no details of growth conditions or the medium used. Bornstein & Barker (1948) found that *Cl. kluyveri* required, as carbon source for growth, ethanol plus acetate, propionate or butyrate and no other compounds were found which would replace these.

Table 8. *Flavin contents of some micro-organisms*

Numbers preceding names of organisms refer to positions in Table 7.

Organism	Total flavin (μm-moles/g. dry wt.)	Flavin in each fraction (%)			FAD content (μm-moles/g. dry wt.)	Calculation method
		Riboflavin	FMN	FAD		
1. *Rhodospirillum rubrum*	30	7	38	55	16	1
2. *Micrococcus lysodeikticus*	40	0	28	72	27	1
3. *Saccharomyces cerevisiae* (aerobic)	100*	0	47	53	52	2
4. *Desulphovibrio desulphuricans*	116	0	21	79	92	1
5. *Azotobacter agile*	125	0	47	53	67	2
6. *Bacillus subtilis*	125 (20)†	0	41	59	74	2
7. *Saccharomyces cerevisiae* (anaerobic)	135*	—	—	—	—	2
8. *Bacillus megaterium*	160	0	43	57	92	2
9. *Lactobacillus casei*	180	0	55	45	81	2
10. *Pseudomonas* OD1	200	8	41	51	104	1
11. *Escherichia coli*	245 (346)‡	0	67	33	81 (80)§	2
12. *Chlorobium thiosulphatophilum*	265	4	46	50	132	1
13. *Lactobacillus helveticus*	270	2	68	30	81	2
14. *Propionibacterium pentosaceum*	275	7	49	44	121	1
15. *Clostridium welchii*	290	5	40	55	160	2
16. *Pseudomonas stutzeri*	310	6	34	60	185	2
17. *Pseudomonas aeruginosa*	360	2	38	60	217	2
18. *Clostridium sporogenes*	460	3	25	72	330	2
19. *Clostridium skatologenes*	480	13	33	54	260	2
20. *Clostridium butyricum*	580 (490)§	2	31	67	390	2
21. Rumen organism LC1	1480	2	25	73	1080	2
22. *Clostridium kluyveri*	2930 (1830)§	2	25	73	2140	2
23. *Thiobacillus denitrificans*	—	0	45	55	—	—

* Values between 96 and 245 μm-moles/g. dry wt. have been reported by various authors (Ingram, 1955). DeLuca *et al.* (1956) found an FAD content of 82 μm-moles/g. dry wt.

† Pett (1935). ‡ Yagi & Mitsuhashi (1951). § DeLuca *et al.* (1956).

In the present work cultures were harvested in the late log phase of growth, as judged roughly either by the rate of gas evolution where this occurred or by the visual appearance of the culture. With several organisms more than one culture was examined and in all such cases the quantitative results showed good agreement (e.g. the data for LC 1 in Tables 5 and 8). However, the comparison of aerobically and anaerobically grown *Sacch. cerevisiae* and the effect of hexanoate on certain organisms (see below) show that growth conditions may have a marked effect upon flavin content and this may account for some of the discrepancies between the results of different authors.

Hexanoate-oxidizing organisms. In view of the association of a high flavin content with the fermentative production of HVFA, it appeared of interest to examine the flavin contents of organisms oxidizing such acids. Accordingly, the three hexanoate-oxidizers, *Pseudomonas* strains CO 1 and CO 2 and *Bacillus sphaericus* CO 8, were isolated and studied.

When grown with hexanoate as carbon source, the two *Pseudomonas* strains contained approximately the same amount of flavin as *Ps. stutzeri* and *Ps. aeruginosa* but appreciably less than the HVFA-producers (Table 9). *B. sphaericus* grown on hexanoate also contained less than the HVFA-producers, but about three times as much as the other *Bacillus* species examined. When other carbon sources were used a marked response to growth on hexanoate was revealed. The wet cell pastes of glucose-grown cells of *Pseudomonas* CO 1 and CO 2 were much paler than hexanoate-grown cells, which are a light-buff colour, and the flavin content was less than one-half of that of hexanoate-grown cells. *B. sphaericus* does not utilize glucose as carbon source but similar effects were observed with acetate-grown cells.

In hexanoate-grown cells of all three organisms a high proportion of the total flavin was FAD. The proportion of FAD in cells grown on other carbon sources was determined with only one organism, namely glucose-grown *Pseudomonas* CO 1, and was much lower than with hexanoate-grown cells.

These results could be completely correlated with the rate of oxygen consumption of washed cells in the presence of hexanoate after growth on the different carbon sources. With all three organisms, the oxidation of hexanoate by washed cells appears to be dependent upon its presence during growth (Table 10). The results should, however, be interpreted with caution. The organisms grow more slowly on hexanoate than on other carbon sources and hexanoate-grown cells were therefore harvested after longer periods of growth. It is notable that with glucose-grown cells of the two *Pseudomonas* strains not only the rate of hexanoate oxidation, but also the rate with glucose and the endogenous rate are less than with hexanoate-grown cells. Similar remarks apply to acetate-grown cells of *B. sphaericus*. The apparent adaptation to hexanoate oxidation might therefore merely be one manifestation of a generally lower metabolic activity of cells grown on substrates other than hexanoate. However, with all three organisms the response of hexanoate oxidation was considerably greater than the response of glucose or acetate oxidation. It therefore seems reasonable to conclude that the ability to oxidize hexanoate and the formation of flavin, particularly FAD, are adaptive, though whether this is due to induced synthesis or the selection of mutants remains an open question.

Application of the method to other materials. No difficulties were encountered when the method was applied to guinea-pig liver, which was first homogenized for 3 min. with 4 ml. of 12·5% trichloroacetic acid/g. of wet liver and then extracted in the usual way. The following results were obtained: total flavin, 352 μm-moles/g. dry wt., distributed as riboflavin 0%, FMN 29%, FAD 71%. Values for rat liver calculated from the data of Yagi (1951), assuming the same dry wt. content (24%) as that found for guinea-pig liver, are: total flavin, 453 μm-moles/g. dry wt., distributed as riboflavin 1%, FMN 9%, FAD 90%.

Three plant materials, cabbage leaves, tomato fruits and beetroot, were also tested and were first frozen at $-20°$ to disrupt the cells and then minced before extraction. The method failed with beetroot because the red pigment was still present in the material taken for electrophoresis and spread over the whole of the paper during the attempted separation. Cabbage and tomato both gave rise to a red–brown pigment which separated upon electrophoresis, as was observed with some of the bacteria. The amount of flavin in these two plants was small and the flavin bands were very streaky and incompletely separated, presumably owing to the presence of relatively large amounts of interfering substances. It was possible to identify FMN and FAD in the appropriate bands but the method in its present form appears unsuitable for quantitative work with plant materials.

DISCUSSION

The methods proposed to date for the estimation of the individual flavins in biological materials are of two general types. The method of Yagi (1951) and that described here involve a preliminary separation of the individual flavins by chromatography or electrophoresis, followed by the separate determination of each. In the second type the principal

Table 9. *Flavin contents of hexanoate-oxidizing organisms*

Numbers preceding names of organisms refer to Table 7, where details of growth are given.

Organism	Carbon source for growth	Duration of growth (hr.)	Total flavin (μm-moles/g. dry wt.)	Flavin in each fraction (%)			FAD content (μm-moles/g. dry wt.)	Calculation method
				Riboflavin	FMN	FAD		
24. *Pseudomonas fluorescens* CO1	0·1% Sodium hexanoate	66	330	0	29	71	228	2
	1% Glucose	48	158	2	54	44	69	2
25. *Pseudomonas fluorescens* CO2	0·1% Sodium hexanoate	120	387	0	25	75	256	2
	1% Glucose	45	108	—	—	—	—	2
26. *Bacillus sphaericus* CO8	0·1% Sodium hexanoate	120	432	0	31	69	291	2
	1% Sodium acetate	90	128	—	—	—	—	2

Table 10. *Oxidative properties of hexanoate-oxidizing organisms*

Washed cell suspensions and determinations of dry weight were made as for examination of flavin contents. Q_{O_2} (μl. of O_2 uptake/mg. dry wt. of cells/hr.) was measured with Warburg manometers containing (in a total reaction volume of 2 ml.) 1 ml. of 0·1M-potassium phosphate, pH 6·8, washed cells as indicated and substrate. Substrates, 10 μmoles of sodium acetate, 10 μmoles of sodium hexanoate or 5·6 μmoles of glucose, originally in 0·1 ml. in the side arm, were added at zero time; 0·2 ml. of 10% KOH was in the centre well. Rates of O_2 uptake were measured over the period 0–20 min. except with acetate-grown *B. sphaericus* CO8 and glucose-grown CO1, where they were measured over the periods 0–30 and 0–60 min. respectively.

Organism	Carbon source during growth	Q_{O_2}				Corrected Q_{O_2}			Wt. of organism/ manometer (mg. dry wt.)
		No substrate (*a*)	With hexanoate (*b*)	With glucose (*c*)	With acetate (*d*)	With hexanoate (*b* − *a*)	With glucose (*c* − *a*)	With acetate (*d* − *a*)	
Pseudomonas CO1	Hexanoate	10·2	59·8	26·6	—	49·6	16·4	—	14·4
	Glucose	5·6	6·7	12·7	—	1·1	7·1	—	13·4
Pseudomonas CO2	Hexanoate	10·3	67·8	49·7	—	57·5	39·4	—	4·7
	Glucose	1·5	2·7	29·2	—	1·2	27·7	—	7·8
Bacillus sphaericus CO8	Hexanoate	62·5	102·5	—	92·3	40·0	—	29·8	9·6
	Acetate	14·3	22·0	—	37·1	7·7	—	22·8	10·8

flavins are not separated from each other but are estimated by differential fluorimetry (Bessey *et al.* 1949) or by a combination of differential fluorimetry with the enzymic assay of FAD (DeLuca *et al.* 1956). Both types of method are open to criticism and it is questionable whether any method is precise. Those based on separation procedures, although ensuring greater specificity and freedom from interference by contaminating materials, involve assumptions about the losses in the stages before the actual assay. On the other hand, methods based on differential fluorimetry and enzymic assay entail fewer preliminary manipulations but presume the identity of the materials being assayed and give less control over interfering substances.

Since little information was available about the flavins of micro-organisms, the present rather laborious procedure was adopted as it enabled the identity of the individual flavins to be established with reasonable certainty. The method is not strictly quantitative and the influence of factors such as age of culture on flavin content has not been investigated. The emphasis has been on a semi-quantitative survey of a number of different physiological types, and for these reasons the deductions drawn from the quantitative data are limited to a few general conclusions.

The principal outcome of the present experiments is the demonstration that FMN and FAD occur in all the micro-organisms examined, and that quantitatively these are the most important flavins. The correlation between a high FAD content and the production of HVFA is also of interest. It suggests that FAD is involved in the metabolism of these acids, and this view is supported by the observations with the hexanoate-oxidizing organisms; not only the flavin content but also the capacity to oxidize hexanoate is greatly increased by growth on this substrate.

There exists at first sight an anomaly in the results obtained with the hexanoate-oxidizers. Even after growth on hexanoate there is much less flavin in these organisms than in either *Cl. kluyveri* or LC1, the two most active HVFA-formers. This difficulty can be resolved on closer consideration. The energy-yielding mechanisms of these last two organisms are not fully understood, but it is evident that the fermentation of lactate by LC1 and of ethanol by *Cl. kluyveri* cannot yield more than a few molecules of adenosine triphosphate (ATP) for each molecule of hexanoate or butyrate produced. By contrast, the complete oxidation of hexanoate by the currently accepted route (Mahler, 1953; Lynen, 1955) would yield some 50 molecules of ATP. The oxidation of a given amount of hexanoate would therefore be expected to support the synthesis of far more cell material than the formation of the same amount by fermentation. In this way, the low flavin contents of the hexanoate-oxidizers relative to the HVFA-producers can be reconciled with the view that flavins are concerned in the metabolism of HVFA *in vivo*.

Work on systems *in vitro* has already established the enzymic role of flavins in fatty acid metabolism. A number of distinct acyl dehydrogenases have been isolated from pig liver and all contain FAD (Crane, Hauge & Beinert, 1955). One of these enzymes, the butyryl dehydrogenase, is specific for the coenzyme A thioesters of the C_4–C_8 fatty acids and is a green cuproflavoprotein (Mahler, 1954).

In a stimulating review, Kaplan (1955) has suggested that, in anaerobes, phosphorylation to yield ATP might be coupled with electron transfer from diphosphopyridine nucleotide to flavoprotein or between two flavoproteins of different oxidation–reduction potentials. Such a system may well operate in HVFA-producers with butyryl dehydrogenase as the electron acceptor (Barker, 1956), and the present work emphasizes the possible importance of such reactions in strict anaerobes.

The present survey has been confined to the intracellular flavins of *Sacch. cerevisiae* and various species of bacteria. Under certain conditions several micro-organisms excrete flavins into the medium (Robinson, 1951) and outstanding in this respect is *Eremothecium ashbyi*. When grown aerobically on glucose–peptone medium both the mycelium of this organism and the medium contain several times more flavin than *Cl. kluyveri* (Yagi, Matsuoka, Kuyama & Tada, 1956). There appears to be no correlation in this case between a high flavin content and the metabolism of HVFA. However, the fact that so much flavin, mostly riboflavin, is excreted into the medium and that crystals of riboflavin have been observed in the mycelium, suggests that in such organisms the synthesis of flavin is not controlled by the requirement of the cell for flavoproteins.

SUMMARY

1. The separation of riboflavin, flavin mononucleotide (FMN) and flavinadenine dinucleotide (FAD) by paper electrophoresis in 0·025 M-ammonium formate–formic acid buffer, pH 4·6, is described. Recoveries of flavin from the paper are about 90%.

2. The use of 10% trichloroacetic acid for 5 min. at room temperature has been selected as the most convenient procedure for extracting flavins from microbial cells. Flavins so extracted are obtained in a form suitable for electrophoresis by fractionation with a Florisil column giving 80% recovery.

3. These methods have been combined in an examination of the flavins present in a range of micro-organisms. FMN and FAD have been demonstrated in all organisms examined. Very little, if any, free riboflavin is present and no other flavin derivatives were detected.

4. Flavin contents varied from 30 to 2930 μ-moles/g. dry wt. with *Rhodospirillum rubrum* and *Clostridium kluyveri* respectively. High flavin contents are associated with the fermentative formation of butyrate and higher volatile fatty acids.

5. With three hexanoate-oxidizing organisms isolated from soil, the oxidation of hexanoate is adaptive and is associated with a higher flavin content.

6. The proportions of flavin present as FMN and FAD vary widely from 21% of FMN and 79% of FAD with *Desulphovibrio* to 68% of FMN and 30% of FAD with *Lactobacillus helveticus*. Those organisms oxidizing hexanoate or producing higher volatile fatty acids contain a high proportion of FAD.

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Studies on the Particulate Components of Rat Mammary Gland

4. THE DISTRIBUTION OF RIBONUCLEIC ACID*

By T. F. SLATER† and D. N. PLANTEROSE
Department of Biochemistry, University College London

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Numerous investigations in the past few years have emphasized the relationship between the ribonucleic acid (RNA) content of a tissue and the rate of protein synthesis in that tissue (see Brachet, 1955). Unlike deoxyribonucleic acid (DNA), RNA is not localized in any one specific intracellular site, but is found to a greater or less extent in the nuclear, the mitochondrial, the microsomal and the soluble fractions of the cell.

Earlier studies on the mammary gland (Greenbaum & Slater, 1957*a*, *b*) have shown that the rapid increase in protein synthesis which occurs at parturition is accompanied by a similar change in the RNA content of the tissue. It was therefore of interest to determine the distribution of RNA in the mammary gland at various stages in the lactation cycle, to see if the increased content of RNA represents a generalized increase in the RNA content of all the separate intracellular fractions, or is the result of an outstanding increase in one particular intracellular fraction. This paper describes the intracellular distribution of RNA in the rat mammary gland at three stages in the lactation cycle: at the end of pregnancy, on the third day of lactation, and on the eighteenth day of lactation.

METHODS

Adult, female albino rats undergoing their first pregnancy or lactation were used in this investigation. For the lactating groups only animals with a litter of six to nine were used. The animals weighed approx. 200 g. For the late-pregnancy group, animals were selected whose appearance indicated a proximity to parturition. The length of the pregnancy was assessed more accurately by examination of the foetuses. These examinations indicated that of the four animals used in this group, one was on the eighteenth day, two were on the nineteenth day and one was on the twentieth day of pregnancy.

Animals were killed by dislocation of the cervical vertebrae and the lower abdominal glands were quickly dissected and placed in ice-cold isotonic sucrose (0·25M). Tissue suspensions (1:10) of mammary gland were prepared as described previously (Greenbaum & Slater, 1957*a*); the time of homogenization was kept constant for each.

The tissue suspensions in 0·25M-sucrose (1:10) were separated into five fractions by differential centrifuging. The procedure was as follows.

(*a*) 'Nuclear' fraction. This fraction (N) was obtained by centrifuging the homogenate at 2000 rev./min. for 10 min. at 0° in a MSE Angle 13 refrigerated centrifuge. The sediment was resuspended in 10 ml. of cold sucrose and respun under the same conditions. A pipette with a bent tip was used for separating the supernatant from the sediment in this and all other cases. The two supernatant suspensions were mixed and used for the isolation of the 'large-particle' fraction.

(*b*) 'Large-particle' fraction (M). The supernatant suspension from (*a*) was spun at 12 000 rev./min. for 15 min. in a MSE Angle 13 centrifuge. The sediment was resuspended in 10 ml. of cold sucrose and resedimented at the same speed. The centrifuge constants for this stage were (de Duve & Berthet, 1953):

$r_{(min.)} = 7{\cdot}5$ cm.; $r_{(max.)} = 12{\cdot}5$ cm.;
temp., 0°; $g_{(max.)} = 14\,500$.

(*c*) 'Intermediate-particle' fraction (I). The supernatant from (*b*) was spun at 13 000 rev./min. for 45 min. in a MSE Angle 13 centrifuge. The constants for this stage were:

$r_{(min.)} = 6{\cdot}5$ cm.; $r_{(max.)} = 12{\cdot}5$ cm.;
temp., 0°; $g_{(max.)} = 17\,000$.

(*d*) 'Small-particle' fraction (U). A volume (26 ml.) of the supernatant suspension from (*c*) was spun in a Spinco preparative centrifuge model L at 40 000 rev./min. for 30 min. The centrifuge constants for this stage were:

$r_{(min.)} = 3{\cdot}8$ cm.; $r_{(max.)} = 8{\cdot}1$ cm.;
temp., 0°; $g_{(max.)} = 144\,700$.

(*e*) 'Soluble' fraction. The supernatant solution from (*d*) is referred to as the 'soluble' fraction (S).

Fractions (N), (M) and (I) were finally suspended in 10 ml. of water; fraction (U) was suspended in 5 ml. of water.

Each fraction was analysed for total protein by the method of Lowry, Rosebrough, Farr & Randall (1951), ox-serum albumin being used as standard. The total protein recovered in the separate intracellular fractions was approx. 90% of the whole homogenate value. This low recovery probably results from the high fat content of the homogenate, which produces elevated extinction values owing to opalescence. This fat is to a large extent removed by solidification during the first centrifuging at 0°. The percentage of protein in the various fractions is therefore expressed in Table 1 as the percentage of the total protein recovered in the five fractions, and not as a percentage of the whole homogenate value. Lactose was estimated in the

* Part 3: Greenbaum & Slater (1957*c*).

† Beit Memorial Fellow.

homogenates as described by Greenbaum & Slater (1957*a*). RNA was determined by a modification (Greenbaum & Slater, 1957*b*) of the procedure of Schneider (1945). The sample was treated with trichloroacetic acid (final concentration, 10%, w/v) in the cold to precipitate proteins and nucleic acids. The sediment was then carefully washed once with 5% (w/v) cold trichloroacetic acid and three times with cold 1% trichloroacetic acid. During each wash the sediment was well dispersed with a stirring rod. The nucleic acids were then extracted with two successive treatments of 5 ml. of 5% trichloroacetic acid at 90° for 15 min. and RNA was estimated on the combined extracts. In this investigation, measurements were made both at 520 and 670 mμ. The few extracts giving a low E_{670}/E_{520} ratio were corrected for the presence of sucrose as described by Slater (1958). That the method of washing used in the RNA estimations successfully eliminated sucrose was indicated by high ratios of E_{670}/E_{520} (see Slater, 1958). The recoveries of RNA in the five fractions totalled 96±4% of the homogenate values.

As with the total protein results, the percentage of RNA sedimented in the various fractions is expressed with respect to the total 'recovered' in the five fractions, and not with respect to the whole-homogenate values. Results have been calculated in terms of ribonucleic acid phosphorus (RNA-P).

RESULTS

Results of the investigation are shown in Table 1. It can be seen that, as found earlier (Greenbaum & Slater, 1957*b*), the RNA-P content of the mammary gland increases sharply at parturition and increases further as lactation proceeds. Although this increase is in part a reflexion of a generalized increase in all the intracellular fractions, the increase in the total amount of the 'intermediate-particle' fraction RNA-P is much greater than the rise in any other fractions. Similarly, with total protein the most extensive change occurs in the 'intermediate-particle' fraction.

The 'specific concentration' of RNA-P (i.e. the RNA-P content of a fraction divided by the total protein content of that fraction) is also shown in Table 1. It is greatest in the 'small-particle' fraction, although the 'nuclear' fraction shows the greatest increase from late pregnancy to late lactation.

DISCUSSION

Before discussing the significance and interpretation of the results obtained in this investigation, the nature of the fractions isolated and the efficiencies of the procedures used for estimating protein and RNA-P will be considered.

Composition of the fractions isolated

The fraction spun down at approximately 6000 *g* min. in isotonic sucrose is conventionally termed the 'nuclear' fraction. In this investigation, however, owing to the difficulties of homogenizing mammary gland tissue, cell nuclei are to a

Table 1. *Distribution of total protein and of ribonucleic acid in the rat mammary gland*

All values quoted in this table are means obtained from four animals (±S.E.M.). For preparation of fractions N, M, I, U and S see the Methods section. H represents the whole homogenate.

State of gland	Total protein in H (g./100 g. of wet tissue)	Total protein in fraction (%)					Total RNA-P in H (mg./100 g. of wet tissue)	RNA-P in fraction (%)					RNA-P/total protein (mg. of RNA-P/100 g. of wet tissue / g. of total protein/100 g. of wet tissue)				
		N	M	I	U	S		N	M	I	U	S	H	N	M	I	U	S
Late pregnancy	6·4± 1·1	6·5± 1·1	14·9± 2·1	4·8± 0·4	7·0± 0·9	66·7± 1·4	6·4± 1·5	4·5± 0·7	11·0± 0·5	11·0± 2·1	21·5± 3·9	51·7± 6·9	1·02± 0·17	0·75± 0·25	1·06± 0·33	1·42± 0·49	4·53± 1·54	0·91± 0·14
Lactating (3 days)	12·3± 1·2	4·9± 0·5	12·4± 0·6	7·2± 0·6	7·4± 0·4	68·5± 1·8	20·7± 3·4	4·5± 0·7	14·5± 2·1	21·5± 1·7	27·0± 3·0	31·7± 2·4	1·44± 0·24	1·11± 0·01	1·47± 0·26	3·33± 1·00	4·20± 1·20	0·58± 0·13
Lactating (18 days)	14·1± 0·5	5·0± 0·6	10·6± 0·7	9·0± 0·1	9·0± 0·6	66·2± 1·2	47·2± 3·9	5·5± 0·7	11·7± 0·8	24·5± 1·7	30·0± 1·8	27·7± 2·0	33·3± 1·20	4·33± 1·01	4·10± 0·52	4·10± 0·44	12·08± 0·75	1·29± 0·17

certain extent ruptured during homogenization, with a consequent loss of contents. Further, there is much greater content of cell debris in a mammary-tissue suspension than is usual for liver and kidney suspensions. The 'nuclear' fraction in this investigation cannot therefore be considered to represent the nuclei as a class of intracellular particle, but rather is composed of a mixture of unbroken nuclei and a large amount of connective tissue and other cell debris. It is likely, by consideration of the centrifuging technique used, that some unbroken nuclei remain in the supernatant fraction after removal of the 'nuclear' fraction and are subsequently sedimented in the 'large-particle' fraction at 200 000 *g* min. This would be expected to increase the percentage of total protein found associated with the 'large-particle' fraction. The 'large-particle' fraction was in fact found to possess some DNA-P, but the extent to which this is the result of adsorption of DNA-P liberated from ruptured nuclei or of the sedimentation of whole unbroken nuclei is not known. As the total protein content of the 'nuclear' fraction is only some 5% of the total homogenate value (see Table 1), the error introduced into the total protein content of the 'large-particle' fraction by the presence of some 10% of the 'nuclear' fraction will be very small.

The 'large-particle' fraction contains the greater part of the mitochondrial fraction in the mammary gland. This fraction in rat mammary tissue covers a wider range of size than in liver: sedimentation of cytochrome oxidase activity in mammary gland increases almost linearly with increasing *g* min. such that at 200 000 *g* min. only some 60% of the total homogenate activity is sedimented.

The 'intermediate-particle' fraction contains the remnants of the particles containing cytochrome oxidase and succinic oxidase plus, presumably, the larger members of the microsomal class, if one defines a microsome as a particle rich in RNA relative to protein. It is not possible to classify microsomal material on a glucose 6-phosphatase basis as is done in the liver (Hers, Berthet, Berthet & de Duve, 1951); rat mammary gland tissue possesses no detectable glucose 6-phosphatase activity.

In contrast with the liver, the mammary gland 'soluble' fraction is not largely composed of cell sap, and largely represents the milk retained in the ducts and alveoli after death. This retained fluid is rich in protein (Greenbaum & Slater, 1957*a*) so that direct usage of total soluble-protein values (and also homogenate-protein values) is pointless in calculating RNA-P:protein ratios. Such ratios can, however, be calculated approximately by estimating the percentage of milk retention and by using the percentage of protein in retained fluid estimated earlier (Greenbaum & Slater, 1957*a*). Such corrections are only very approximate as the percentage of protein in retained fluid is not strictly constant for each stage of the lactation cycle, but varies rather widely according to such factors as the season, the diet and the size of the litter.

Distribution of ribonucleic acid phosphorus

The changes in homogenate RNA-P are similar to those previously reported by Greenbaum & Slater (1957*b*). The RNA-P values are lower than theirs, but this is probably the result of seasonal variations in gland composition. All the observations reported in this investigation were made in a period of 1 month, whereas those of Greenbaum & Slater (1957*b*) were spread throughout the whole year. The late-pregnancy group values are much lower than previously found by Greenbaum & Slater but, as mentioned in the Methods section, animals used varied between the eighteenth and twentieth day of pregnancy; it is known that the composition of the gland changes rapidly at the end of pregnancy.

The general pattern for the distribution of RNA-P is closely similar to that described for liver, kidney and other tissues (see Hogeboom & Schneider, 1955). The bulk of the cell RNA-P is in the 'small-particle' and 'soluble' fractions. There are, however, very significant alterations in this generalized pattern between the various stages of lactation studied.

In late pregnancy, for instance, 50% of the total RNA-P is in the 'soluble' fraction. This is much higher than the 16% reported for the soluble fraction of mouse liver (Schneider, Hogeboom & Ross, 1950). This high value (51·7% on the twentieth day of pregnancy) falls rapidly and significantly ($P < 0{\cdot}05$) over parturition and throughout lactation to some 28% on the eighteenth day of lactation. At the same time the percentage of RNA-P in the 'small' and 'intermediate-particle' fractions is rising, although the percentage of RNA-P in the 'nuclear' and 'large-particle' fractions is remarkably uniform. The percentage of RNA-P in the 'intermediate-particle' fraction is more than doubled from late pregnancy to late lactation ($P < 0{\cdot}01$). The results therefore show that as lactation advances and synthesis of milk rises towards its maximum, the distribution of RNA-P in the mammary gland cells changes, the emphasis moving from the 'soluble' to the 'small' and 'intermediate' particulate fractions. (These last two fractions may be termed the smaller-particle fractions of the cell.)

Distribution of protein

Table 1 shows that the percentages of total protein sedimented in the various fractions show little change over the lactation cycle, with the

exception of the 'intermediate-particle' fraction which shows an increase of almost 100% from late pregnancy to late lactation ($P<0.02$); the other values show little change in spite of a large increase in total protein.

These values are, however, based on the total protein of the homogenate, which, as previously mentioned, includes the protein of the retained fluid. Considerable changes in the intracellular fractions may therefore be hidden by the very high protein contribution of the retained fluid. The protein sedimented in the 'large', 'intermediate' and 'small-particle' fractions has therefore been combined as 'particulate protein', and the percentage of this total value sedimented in each of the three subfractions contributing to it has been calculated. The values are shown in Table 2. It can be seen that considerable alterations occur in the distribution of protein among the intracellular particulate fractions over the lactation cycle. As protein synthesis commences on a large scale at parturition and rises to a peak towards the end of lactation, there is an increase in the relative concentration of protein material in the smaller particulate fractions of the cell. Since the total amount of 'large-particle' material increases over parturition and throughout lactation (Greenbaum & Slater, 1957*b*), results in Table 2 imply that the smaller-particle fractions increase even more rapidly over the lactation cycle.

Ratio of ribonucleic acid phosphorus:protein

The ratio RNA-P:total protein for the various fractions is shown in Table 1. The threefold increase in this ratio for the whole homogenates is again distorted by the inclusion of retained fluid protein. This objection does not, however, apply to the particulate fractions, where it can be seen that RNA-P is highly concentrated relative to protein in the 'intermediate-particle' and 'small-particle' fractions. In the 'small-particle' fraction the ratio in late lactation is similar to that obtained by Keller, Zamecnik & Loftfield (1954) for rat-liver microsomes.

By the use of values for milk composition previously published (Greenbaum & Slater, 1957*a*), the distribution of protein in the various fractions and the ratio of RNA-P:total protein for the various fractions have been recalculated on a milk-free-tissue basis. The results are shown in Table 3. As mean values for milk composition and percentage milk retention have been used no standard errors can be calculated. It can be seen that the distribution of total protein is not substantially altered by the correction for milk. The percentage of protein in the 'soluble' fraction is naturally decreased with a consequent increase in the percentage of total protein associated with the particulate fractions. The general pattern of distribution is, however, unchanged.

Table 2. *Distribution of protein in the 'large', 'intermediate', and 'small-particle' fractions of rat mammary gland*

All values quoted are means obtained from four animals (±S.E.M.).

State of gland	Protein sedimented in each fraction (% of total protein in M+I+U) 'Large-particle' fraction (M)	'Intermediate-particle' fraction (I)	'Small-particle' fraction (U)
Late pregnancy	55·4±3·1	18·5±2·6	25·9±2·6
Lactation (3 days)	45·9±0·9	26·8±1·7	28·1±0·8
Lactation (18 days)	38·8±2·0	30·9±1·0	30·6±1·2

Table 3. *Distribution of total protein and the value of ribonucleic acid phosphorus:total protein in milk-free fractions of rat mammary gland*

For preparation of fractions N, M, I, U and S see Methods section.

State of gland	Distribution of protein (% of total protein) N	M	I	U	S	RNA-P:total protein (mg. of RNA-P/100 g. of milk-free tissue / g. of total protein/100 g. of milk-free tissue) H	S
Late pregnancy	9	22	7	9	52	1·5	1·6
Lactating (3 days)	7	15	9	9	60	2·0	1·5
Lactating (18 days)	7	16	13	13	50	5·2	3·1

The ratio of RNA-P:total protein, on the other hand, shows an increase in both the homogenate and 'soluble' fraction; this results from the lowering in the total protein value used in this calculation as a consequence of the removal of milk protein.

SUMMARY

1. The distribution of total protein and ribonucleic acid phosphorus in five fractions of the rat mammary gland have been determined at three stages of the lactation cycle: on the twentieth day of pregnancy and on the third and eighteenth days of lactation.

2. At the end of pregnancy, ribonucleic acid phosphorus is largely localized in the 'soluble' fraction of the mammary gland. Lactation produces a redistribution of total ribonucleic acid phosphorus such that the majority of it is found in the smaller particulate fractions of the tissue suspension.

3. Lactation produces a relative increase in the amount of protein associated with the smaller particulate fractions at the expense of the 'large-particle' fraction. All these fractions, however, show an increase in total amount from late pregnancy to late lactation.

4. The ratio of ribonucleic acid phosphorus to total protein is low in all fractions in late pregnancy but has risen to a high value in the 'small-particle' fraction by the end of lactation.

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The Excretion of Urea, Ammonia and Purine End-products by the Newborn Animal

By ELSIE M. WIDDOWSON, J. W. T. DICKERSON and R. A. McCANCE
Medical Research Council Department of Experimental Medicine, University of Cambridge

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It is usually taught that mammals other than primates, and insects except the Diptera, excrete 80–100% of the uric acid they form only after oxidizing it to allantoin. In dogs in particular, apart from the Dalmatian coach-hound, it would seem that the amount of uric acid in the urine is normally minute, and that, even after injecting the acid, 98% of it enters the urine as allantoin (Best & Taylor, 1955; Evans, 1956). The uric acid excreted by the Dalmatian coach-hound, once thought to be due to a purine metabolism resembling that of primates, is now attributed to there being no mechanism in the renal tubules for reabsorbing uric acid (Friedman & Byers, 1948; Miller, Danzig & Talbott, 1951). Yet Madsen, Earle, Heemstra & Miller (1944) reported finding uric acid infarcts in the kidneys of newborn piglets suffering from a disease which they described as 'acute uremia'. Morrill (1952*a*, *b*) found accumulations of uric acid crystals in the kidneys of piglets which appear to have had a similar if not identical disease.

In another connexion it was noticed in this Department that in newborn puppies urea might form less than 30% of the total N excreted. The turnover of protein in the body has not been so well explored in the newborn period as in adult life, and the information which can be obtained about cellular metabolism from a study of the excretion of the end-products of nitrogenous metabolism in the urine made it seem desirable to examine the excretion of the important end-products, uric acid and allantoin, and also that of urea and ammonia by newborn mammals. Human infants, piglets and puppies were chosen for the investigation.

MATERIAL AND METHODS

Urine formed before birth and passed at the time of birth or very shortly afterwards has been collected from eight full-term babies, five piglets and three puppies. Collections over the whole of the first 48 hr. of life have been made on seven babies, and on the seventh day on six of them. These babies were treated in the normal way and put to the breast every 4 hr. from the second day of life onwards. They obtained 8–15 ml. of colostrum at first, 20–30 ml. by the end of the second day and 300–500 ml. of milk on the seventh day. None had any cow's-milk supplements. A total of eighteen piglets from four litters have been put into metabolism cages as soon as they were born and all their urine was collected for 40 hr. Four of these piglets, one from each litter, were given sow's milk, four an equal volume of water, and four nothing. The liquid was given by stomach tube every 2 hr. as described by McCance & Widdowson (1956). In addition, a fourth piglet from each of three of the litters was given the same quantity of a solution of sodium chloride in water (0·5, 0·7 or 0·9%). A fifth pig from each of these three litters was given salted sow's milk made up so that the concentration of sodium chloride in the water of the milk was 0·5, 0·7 or 0·9%. Puppies, one from each of four litters, have also been fed by stomach tube every 2 hr. Dog's milk was used for the first and sow's milk for the other three. Urine was collected from the puppies as described by McCance & Widdowson (1955). The experiments on the puppies lasted for 24 hr. Collections of urine (24 hr.) from two men and four women, and isolated specimens from three adult pigs and nine dogs, have also been analysed.

All urines were collected under toluene and stored in a refrigerator at 4°. Total nitrogen was determined by the micro-Kjeldahl technique with copper selenite as catalyst, and urea by Lee & Widdowson's (1937) method. Ammonia was estimated either by distilling in a micro-Kjeldahl apparatus at pH 8, with phenol red as indicator, or, where the samples were small, in a Conway unit (Conway, 1950). Results obtained for the same urine by the two methods were found to agree very closely. Uric acid was determined as recommended by King (1946) and allantoin by the method described by Young & Conway (1942). It was found that uric acid gave the same colour reaction as allantoin by this method, 1·0 mg. of uric acid being equivalent to about 0·06 mg. of allantoin. The uric acid content of the urine of newborn infants and adults was sufficient to account for the small amounts of colour which were produced by the allantoin reagents. The values given for allantoin in the urine of adult and newborn pigs have been corrected for the colour due to uric acid.

RESULTS

Table 1 shows the average concentration of nitrogen in the urines of man, the pig and the dog at several stages of development, and the percentage of the total nitrogen excreted as urea. As has been found by others (Folin, 1905*a*; Cathcart, 1907; Barlow & McCance, 1948), urea accounted for 80–90% of the total nitrogen in the urine of healthy adult men and dogs. It was always below 80% in pigs. In all three species the figure was lower in the newborn period than in the adult, and in the puppy it was only 20–30% just before and in the 12 hr. after birth. The newborn puppies' urine contained 10–25% of protein nitrogen, but even when the results are expressed on a protein-free basis urea still accounted for only about 30% of the nitrogen in the urine passed at birth and during the first 12 hr. afterwards. The proportion of urea increased rapidly, and during the second 12 hr. of the 24 it comprised 65% of the total nitrogen, or 71% on a protein-free basis.

The newborn of all three species excreted a higher proportion of their nitrogen as ammonia than did the corresponding adults (Table 2), and the ammonia coefficient was always highest in the urine formed in the uterus and passed at birth.

The figures for uric acid and allantoin are shown in Table 3. The results on adult men and dogs confirm those of earlier workers; newborn piglets and puppies, like human babies (Barlow & McCance, 1948), excreted more of the total nitrogen as uric acid than did the adults, but in all three species the excretion of uric acid was more variable than that of urea or ammonia. The proportion of the total nitrogen present as uric acid in the

Table 1. *Percentage of the total nitrogen excreted as urea by newborn babies, piglets and puppies compared with adults of the same species*

Standard deviations of the percentages have been given where the numbers were 6 or more. Figures in parentheses denote number of observations.

	Man		Pig		Dog	
Nature of urine	Total N (mg./100 ml.)	Total N as urea (%)	Total N (mg./100 ml.)	Total N as urea (%)	Total N (mg./100 ml.)	Total N as urea (%)
Formed *in utero* and passed at birth	80 (8)	72±4·8	196 (5)	64	308 (3)	23
First 24 hr. after birth	562 (7)	70±5·7	175 (4)	58	545* (3)	28
					886† (3)	65
Second 24 hr. after birth	733 (7)	76±4·6	154 (4)	72	—	—
Seventh day	132 (6)	82±3·5	—	—	—	—
Adult	660 (6)	85±3·7	804 (3)	76	1560 (9)	88±5·1

* 1st 12 hr. † 2nd 12 hr.

Table 2. *Excretion of ammonia by newborn babies, piglets and puppies compared with adults of the same species*

Results for ammonia nitrogen are expressed as percentage of total nitrogen. Standard deviations have been given where the numbers were 6 or more.

Nature of urine	Man	Pig	Dog
Formed *in utero* and passed at birth	10·2±1·55	10·4	15·0
First 24 hr. after birth	7·6±0·40	8·0	14·9*
			8·6†
Second 24 hr. after birth	7·4±0·56	6·2	—
Seventh day	6·7±0·87	—	—
Adult	3·5±0·48	1·9	2·5±0·82

* 1st 12 hr. † 2nd 12 hr.

Table 3. *Excretion of uric acid and allantoin by newborn babies, piglets and puppies compared with adults of the same species*

Results are expressed as percentage of total nitrogen. Standard deviations have been given where the numbers were 6 or more.

	Man		Pig		Dog	
Nature of urine	Uric acid	Allantoin	Uric acid	Allantoin	Uric acid	Allantoin
Formed *in utero* and passed at birth	9·9±3·35	0	2·7	1·5	2·7	27·2
First 24 hr. after birth	9·0±3·57	0	1·1	1·4	1·6*	13·5
					0·6†	4·6
Second 24 hr. after birth	8·3±2·92	0	1·1	1·5	—	—
Seventh day	3·8±1·45	0	—	—	—	—
Adult	2·2±0·81	0	0·7	2·8	0·4±0·07	3·6±0·88

* 1st 12 hr. † 2nd 12 hr.

Table 4. *Effect of food and of sodium chloride on the percentage of nitrogen excreted as urea by piglets in the first 24 hr. of life*

	Percentage of total N excreted as urea			
	Litter 1	Litter 2	Litter 3	Average
Fed on milk	60·3	53·8	59·3	57·8
Given water only	68·8	58·7	67·5	65·0
Fed on milk + NaCl*	55·6	44·2	40·3	46·7
Given water + NaCl*	63·5	53·4	51·1	56·0

* Litter 1 had 0·5%, litter 2 had 0·7% and litter 3 had 0·9% of NaCl.

puppies' urine fell rapidly, so that during the second 12 hr. after birth the figure was little higher than it was in the adult. Although King's (1946) method is not specific for uric acid, it is unlikely that the differences between the newborn and the adult could have been accounted for by interfering substances such as polyphenols or methylxanthines, e.g. caffeine.

The urine formed by puppies in the uterus contained 27% of its nitrogen in the form of allantoin. Again the proportion fell rapidly, so that the urine passed during the second 12 hr. of life resembled adult urine in the relative proportions of the total nitrogen present as uric acid and allantoin.

DISCUSSION

The percentage of the total nitrogen excreted as urea seems generally to be lower in the newborn than in the adult of the same species. This is not due to starvation or to a low-protein diet (Folin, 1905*b*). It may in some species be due partly to the excretion of much nitrogen in the form of protein, but a more important reason is that the balance between anabolism and catabolism is so strongly weighted on the side of anabolism. Tissue proteins are little broken down, and since so much of the protein in the food is used for purposes of growth the formation of urea is small; hence its concentration in the plasma, and consequently the percentage of the urea nitrogen in the urine, remains low. The administration of sodium chloride to piglets has been shown to decrease catabolism of protein (McCance & Widdowson, 1957), and Table 4 shows that it also decreases the percentage of the total nitrogen excreted as urea. McCance & Finck (1947) reported that the ammonia coefficient of the urine of newborn infants was on the whole higher than that of the urine passed by adults. The present results confirm this and extend the observation to other species. The high coefficient is not the result of the pH of the urine being very low, for the pH of the urines passed by human infants at birth (average 6·3) was almost the same as that of the urines passed by the adults (average 6·0). Nor is the high coefficient the result of the kidney at this age being able to form ammonia with great facility, for the evidence which has been collected indicates that glutaminase activity is not so great in the newborn as it is in the adult (Cort & McCance, 1954; Hines & McCance, 1954). The relatively great excretion of ammonia in the new-

Table 5. *Concentration of uric acid in the urine of babies and of piglets fed with milk and receiving nothing*

Results are expressed as mg. of uric acid/100 ml. of urine.

	Human babies	Piglets		
		Receiving milk	Receiving water	Receiving nothing
First 24 hr. after birth	151	5·3	7·3	56·0
Second 24 hr. after birth	185	4·6	6·7	87·0

born period may be partly due to the fact that the titratable acidity is so low (McCance & Finck, 1947) that nearly all the acid to be eliminated by the kidney must be combined with ammonia or fixed base. The main reason for the high coefficient is, however, the small amount of nitrogen, and particularly of urea, in the urine. The total excretion of ammonia/kilogram of body weight or/square metre of surface area is lower in the newborn than at older ages (Widdowson & McCance, 1958). The uric acid infarcts which have been found in the kidneys of ailing newborn piglets are easily explained. They are due to the relatively large amounts of uric acid excreted by these animals in the newborn period, coupled with a small volume of urine which is the outcome of the dehydration from which the animals suffer when they get too little milk. Table 5 shows the concentration of uric acid in the urine of piglets which were given sow's milk by stomach tube, and of other piglets which received the same volume of water, or nothing at all, for the first two days of life. The urine of the starved and dehydrated piglets contained 8–20 times as high a concentration of uric acid as that of the other animals. The urine of normal healthy babies contains an even higher percentage.

Since puppies also excrete a higher proportion of their end-products of purine metabolism as uric acid than do adult dogs, this would seem to be characteristic of the newborn, at least of some species, and to be a sign of incomplete chemical or physiological development—chemical if it is due to the necessary uricase activity's still being undeveloped, physiological if it is due to a very low renal threshold for uric acid. The present investigation does not enable one to distinguish between these two alternatives.

SUMMARY

1. The excretion of urea, ammonia, uric acid and allantoin by newborn babies, pigs and puppies has been studied, and the results have been compared with values for adults of the same species.

2. The percentage of the total nitrogen excreted as urea was lower in the newborn than in the adult of the same species. This has been attributed to the turnover of amino acids being directed towards anabolism at this age.

3. The newborn baby, pig and puppy excreted a higher proportion of their nitrogen as ammonia than did the adults. The reasons for this are discussed.

4. Newborn pigs and puppies excreted more of their purine end-products as uric acid than did the corresponding adults. In the urine formed by puppies in the uterus allantoin contributed a higher percentage of the total nitrogen than did urea. The percentage contributed by allantoin declined rapidly after birth.

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Studies on Zinc Toxicity in the Larvae of the Rice Moth, *Corcyra cephalonica* St.

By K. SIVARAMA SASTRY, R. RADHAKRISHNA MURTY* and P. S. SARMA
University Biochemical Laboratory, Madras-25, India

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Zinc salts fed at relatively high concentrations in the diet have been known to produce deleterious effects. Sutton & Nelson (1938) found that 1 % of zinc as carbonate impaired the growth and general health of rats. Smith & Larson (1946) were, however, the first to study extensively this phenomenon in rats. They established that this condition manifested itself in the following two distinct and unrelated symptoms: (*a*) an anaemia, of the microcytic, hypochromic type, and (*b*) an inhibition of growth. The former syndrome could be prevented by supplementing the diet with low levels of copper, and liver extract could partially reverse the adverse influence on growth. van Reen (1953) found that at the 0·5–0·7 % level, zinc reduced liver catalase and cytochrome oxidase and that copper could counteract this influence of zinc. These studies indicated that toxic levels of zinc interfere with the utilization of copper and thus indirectly with the formation of iron-containing enzymes. van Reen & Pearson (1953) concluded, however, that the toxicity of zinc was not primarily due to disturbances in these enzyme levels. Such a conclusion is supported by the fact that serious impairement of growth occurs before reduction in any of the enzymes studied.

Thus the underlying mechanisms in zinc toxicosis are not fully known. The situation is complex and although the studies of Smith & Larson (1946) have shown that liver extract is beneficial in zinc toxicosis, the factors responsible have not been characterized, nor is anything known concerning zinc toxicity in other species. In this paper, the results of investigations on zinc toxicity in the lepidopterous insect *Corcyra cephalonica* St. are presented. Although this organism is much lower in the evolutionary scale, it shows striking similarities (Sarma, 1955) to higher organisms like the rat in its nutritional requirements and metabolism.

EXPERIMENTAL

Materials. Zinc was added to the larval diets generally as zinc sulphate ($ZnSO_4,7H_2O$) except in the first set of experiments (Table 1), where zinc chloride was used. Both the salts were products of Merck and Co. Ltd.

All the vitamins used in the growth experiments were purchased from Chas. Pfizer and Co., U.S.A.

Deoxyribonucleic acid and ribonucleic acid were commercial products of Schwarz Laboratories Inc., New York, N.Y., U.S.A.

Liver extract was prepared in the laboratory from sheep liver by autolysis followed by digestion with papain (Mistry, Gajjar & Sreenivasaya, 1945) to yield a final solution containing 100 mg. of total solids/ml. A commercial concentrate (product of Teddington Chemical Factory Ltd., Andheri, Bombay) prepared from the same source was also employed in one set of experiments (Table 3). In this extract 1 ml. was equivalent to 15 g. of proteolysed sheep liver. The alkali-stable fraction of liver extract was prepared by adjusting the extract to pH 11–12 and autoclaving it for 30 min. at 120° (Shenoy & Ramasarma, 1955).

Larval diets and growth studies. Young (10–15 days-old) larvae of the rice moth were used in all experiments. The procedure employed in rearing the larvae was essentially the same as in earlier studies (Sivarama Sastry, 1955).

The basal diet consisted of 10 g. of sieved wheat flour contained in 9 cm. Petri dishes. Supplements, whenever added, were mixed well with wheat flour by using 10–20 ml. of water to achieve uniform distribution. The diets were then allowed to dry at 60° overnight and finely powdered. Thirty larvae were usually reared in each dish.

In all investigations, batches of ten larvae were picked from each dish and weighed every week. The growth data were recorded as the average weights of groups of ten larvae in milligrams. A difference of over 10% in larval weights was always reproducible and hence was considered significant. Growth was studied over 2–3 weeks depending upon the initial size of the larvae, the period being such that in each case the life span of the larval stage from the commencement of the experiment was covered. Where possible, larvae of the same weight were used for each group of experiments. When the initial weights were different, separate control groups were included and all growth data were evaluated on the basis of the appropriate control group.

Experiments with ^{65}Zn. In studies with radioactive zinc, 43 μg. of zinc as $^{65}ZnCl_2$ (supplied by the Radiochemical Centre, Amersham, Bucks.), with a total radioactivity of 9000 counts/min., was added to each gram of diet and the diets were prepared as usual. Larvae were allowed to grow for 3 weeks on the usual diets and then given diets containing ^{65}Zn for 2 days. The larvae were cleaned with a fine brush till completely free of adhering diet and ground up with water. Samples were plated out for measurement of

* Present address: Central Food Technological Research Institute, Mysore-2, India.

radioactivity so that each planchet contained between 30 and 40 mg. of dry larval tissue. ^{65}Zn was determined with an end-window Geiger–Müller tube (2·1 mg. of mica/cm.^{2}) attached to a decade-type autoscaler (Panax Equipment, Mitcham, Surrey). All samples were counted with an accuracy of ±2%. No self-absorption corrections were made.

RESULTS

The effects of various levels of dietary zinc on the growth and survival of rice-moth larvae are presented in Table 1.

The various levels of zinc chloride were chosen to determine broadly the range within which zinc is toxic to the organism. It can be seen that at 0·08 and 0·16% levels of zinc chloride (representing respectively 400 and 800 p.p.m. of zinc) there is no marked effect on growth or survival. The latter level is obviously near the threshold beyond which zinc becomes deleterious, since a very marked inhibition of growth with a concomitantly high mortality was obtained with 0·8% of zinc chloride. When the level of zinc chloride was doubled to 1·6% the larvae barely survived for 1 week. The pattern was the same when zinc sulphate was used instead of zinc chloride; in this case the results obtained at a level of 0·1% were similar to those with 0·08% of zinc chloride and 1% was found to be lethal by the end of the third week.

Since Smith & Larson (1946) had shown, in rats, that liver extract could partially reverse the inhibition of growth due to zinc toxicosis it was decided to study whether liver extract was beneficial in the larvae as well. Vitamin B_{12} was also tested at the same time, in view of its importance as one of the anti-anaemic principles of liver extract, and its ability to alleviate the symptoms of metal toxicities, namely, molybdenum toxicity in *Lactobacillus leichmannii* (Daniel & Gray, 1953) and lead anaemia in rabbits (van Klavern & Shrivastava, 1954). The results are summarized in Table 2.

It can be seen that both vitamin B_{12} and liver extract reduced the mortality due to lethal doses of dietary zinc; but liver extract used at a level to provide the same vitamin B_{12} concentration was more effective, suggesting the presence of factors other than this vitamin in liver extract which are involved in this process. It may be seen, however, that in this acute condition, neither liver extract nor vitamin B_{12} restored the growth of the larvae to normal. Subsequently a sublethal, growth-inhibitory level of 0·4% of zinc sulphate was chosen as the most suitable concentration for further experiments.

The effect of liver extract and of the alkali-stable fraction on the growth of the larvae on a diet with 0·4% of zinc sulphate was investigated. The results obtained are shown in Table 3.

It is evident that liver extract reverses the inhibition of growth to a very large extent, and that the same result is obtained with the alkali-stable fraction, which accounts quantitatively for the activity of liver extract. In view of this observation other B vitamins were tried for their effective-

Table 1. *Toxicity of zinc chloride to rice-moth larvae*

Expt. no.	Concn. of $ZnCl_2$ in diet (%)	Wt. of 10 larvae (mg.) At transfer	Week 1	Week 2	Week 3	Mortality at the end of expt. (%)
1	0 (control)	8·8	33	141	257	0
2	0·08	8·6	43	122	276	0
3	0·16	8·9	35	113	235	0
4	0·80	8·2	10	13*	14*	78
5	1·60	8·2	2*	—†	—†	100

* Values recorded for surviving larvae. † No survivors.

Table 2. *Influence of liver extract and vitamin B_{12} on growth and survival of rice-moth larvae in zinc toxicosis*

Supplements to basal diet (10 g. of wheat flour)	Wt. of 10 larvae (mg.) At transfer	Week 1	Week 2	Week 3	Survival at the end of expt. (%)
None (control)	5·1	37	120	244	100
Zinc sulphate (0·1 g.)	5·0	6	4*	2*	7
Zinc sulphate (0·1 g.) + vitamin B_{12} (10 μg.)	4·8	10	12	18*	47
Zinc sulphate (0·1 g.) + liver extract†	5·2	12	19	43	70

* Values recorded for surviving larvae.

† Liver extract prepared from sheep liver (see text) was added to provide 10 μg. of vitamin B_{12} (assayed with *Lactobacillus leichmannii* A.T.C.C. 7830 (Radhakrishna murty & Sarma, 1957).

ness in overcoming the inhibition of growth. The results of a typical experiment are presented in Table 4.

Of all the vitamins tested, only vitamin B_{12} and thiamine were effective, even though the restoration of growth was far from complete. It may be mentioned that the levels of the vitamins in this set of experiments were in excess of the normal nutritional requirements of this organism. The growth responses recorded in Table 4 represent the maximum obtained with the various vitamins. In view of the growth-promoting effect of the alkali-stable fraction of liver extract and the comparative ineffectiveness of the B vitamins it seemed worthwhile to study whether the deoxyriboside fraction of liver extract was involved. For this purpose, the influence of deoxyribonucleic acid was examined, and in the same experiment the influence of ribonucleic acid was also studied.

The results (Table 5) indicate that deoxyribonucleic acid (DNA) is very effective at the 0·5% level, maximal reversal occurring at the 1·0% level. It can be seen further that ribonucleic acid is similarly effective. It would thus appear that the synthesis or metabolic activity of the nucleic acids is adversely affected in zinc toxicosis in this organism and that the activity of the liver extracts might be due also to their content of nucleic acids or nucleotides. However, it seemed possible that DNA might make zinc unavailable to the larvae by chelating with the metal (Neuberg & Roberts, 1949). To investigate this possibility larvae were grown on wheat flour (control diet), wheat flour containing 0·4% of zinc sulphate, or wheat flour supplemented with 0·4% of zinc sulphate and 1·0% of DNA, for a period of 3 weeks as in the experiments recorded in Table 5. At the end of this period larvae from each dish were removed and placed for 2 days on diets containing ^{65}Zn alone or ^{65}Zn and DNA, and their ^{65}Zn content was determined as mentioned earlier.

It may be observed from the results in Table 6 that the same amount of zinc is taken up by the larvae whether zinc was supplied alone or in conjunction with DNA, ruling out the possibility that DNA was making zinc unavailable to the organism.

Table 3. *Influence of liver extract and the alkali-stable fraction on inhibition of growth due to zinc toxicosis*

All diets, except no. 1, contained 0·4% of $ZnSO_4,7H_2O$.

Diet no.	Supplements to 10 g. of basal diet	Wt. of 10 larvae (mg.) At transfer	Week 1	Week 2	Wt. gain (mg./10 larvae)
1	None (control, no zinc sulphate)	16	75	287	271
2	None	17	50	158	141
3	2 ml. of liver extract*	17	61	229	212
4	2 ml. of liver extract (alkali-treated)*	16	69	234	218

* Commercial liver extract (see text).

Table 4. *Influence of vitamins of the B complex on zinc toxicosis in rice-moth larvae*

Unless otherwise indicated all diets contained 0·4% of $ZnSO_4,7H_2O$.

Supplements to 10 g. of basal diet	Wt. of 10 larvae (mg.) At transfer	Week 1	Week 2	Week 3
	Experiment *A*			
None (control; no zinc sulphate)	6·7	46	138	237
None	6·6	29	53	127
Vitamin B_{12} (10 μg.)	5·9	34	82	143
Thiamine (1 mg.)	7·8	31	84	157
Pyridoxine (1 mg.)	6·1	29	58	112
Calcium pantothenate (10 mg.)	6·2	29	57	124
Inositol (10 mg.)	6·6	29	56	120
Nicotinic acid (2 mg.)	6·4	30	58	126
Biotin (10 μg.)	6·1	28	55	125
p-Aminobenzoic acid (10 mg.)	6·5	33	55	119
	Experiment *B*			
None (control; no zinc sulphate)	15·0	54	140	253
None	14·0	34	72	111
Folic acid (10 μg.)	14·7	37	68	117
Riboflavin (10 μg.)	13·7	32	62	116
Choline chloride (2 mg.)	16·0	36	63	110

Table 5. *Influence of deoxyribonucleic acid and ribonucleic acid on zinc toxicity in rice-moth larvae*

Unless otherwise indicated, all diets contained 0·4% of $ZnSO_4,7H_2O$.

Supplements to 10 g. of basal diet	Wt. of 10 larvae (mg.) At transfer	Week 1	Week 2	Week 3	Wt. gain (mg./10 larvae)
None (control; no zinc sulphate)	4·0	36	125	198	194
None	4·0	22	60	103	99
0·5% of deoxyribonucleic acid	3·6	21	106	173	169·4
1·0% of deoxyribonucleic acid	3·6	32	132	189	185·4
2·0% of deoxyribonucleic acid	3·5	32	114	184	180·5
1·0% of ribonucleic acid	6·0	42	110	197	191

Table 6. *Influence of deoxyribonucleic acid on uptake of* ^{65}Zn *by tissues of the rice-moth larvae in zinc toxicosis*

Initial larval diet	Uptake of ^{65}Zn by larval tissues (counts/min./100 mg.) With ^{65}Zn alone	With ^{65}Zn and DNA*
Control (no zinc sulphate)	258·8	245·6
0·4% of zinc sulphate	155·2	145·5
0·4% of zinc sulphate and 1·0% of DNA	181·5	179·5

* DNA was added, in each, to provide the same ratio of zinc to DNA as in the growth experiments.

DISCUSSION

The present investigation has shown that in the larvae of the rice moth, *Corcyra cephalonica* St., the inhibition of growth induced by 0·4% of zinc sulphate is partially reversed by either whole-liver extract or its alkali-stable fraction. None of the B vitamins tested was as effective. Thus it would appear that in zinc toxicosis the vitamin status of this organism is mostly unaffected. The behaviour of vitamin B_{12} in reducing the mortality but not the inhibition of growth could be explained on the basis of its possible involvement in nucleic acid synthesis in the larvae, particularly in view of the influence of the nucleic acids on zinc toxicity. It is also possible that the vitamin B_{12} status of the larvae is affected by lethal levels of dietary zinc but not by the lower ones.

The results presented in Table 6 show that DNA, at a level at which it reverses the inhibition of growth completely, does not affect the assimilation of ^{65}Zn to any extent, as the uptake of ^{65}Zn by each of the three groups of the larvae is the same both in the presence and in the absence of DNA. It is likely that in zinc toxicosis in this organism the need for either the nucleic acids as such, or for some of the structural constituents of the DNA molecule, is increased and that this situation brings about the observed inhibition of growth. Further studies would be necessary to determine how the synthesis or metabolism of these compounds is affected in zinc toxicity.

SUMMARY

1. The levels at which dietary zinc becomes toxic to rice-moth larvae have been determined. At lethal levels of zinc, supplementation of the diet with vitamin B_{12} or liver extract checked mortality.

2. None of the B vitamins, except thiamine and vitamin B_{12}, was effective in reversing the inhibition of growth induced by 0·4% of zinc sulphate. Vitamin B_{12} was more effective in prolonging the survival of the larvae than in promoting growth in this condition.

3. Liver extract, as well as its alkali-stable fraction, partially reversed the inhibition of growth due to zinc toxicity.

4. Deoxyribonucleic acid and ribonucleic acid at 0·5–1·0% levels in the diet reversed the inhibition of growth due to zinc toxicity completely. Dietary DNA did not influence the uptake of ^{65}Zn from the diet. The results are discussed.

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The Lipids of Ox Compact Bone

BY A. A. LEACH
The British Gelatine and Glue Research Association, 2a Dalmeny Avenue, Holloway, London, N. 7

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Pikular (1955) determined the cholesterol content of compact bone after demineralization with hydrochloric acid. By the digitonide method, the cholesterol content of the organic matter (osseocollagen) was found to be 0·0028–0·0040 g. of cholesterol/100 g. of dry ash-free protein. He also prepared procollagen by the method of Orekhovich, Tustanorskiĭ, Orekhovich & Plotinkora (1948) and showed cholesterol to be present, but did not measure the quantity. Pikular (1955) pointed out that the cholesterol content of the collagenous material was very similar to that found in myostroma by Nikol'skaya (1953) and concluded that the cholesterol was present in the form of loose and firm complexes with protein.

Spector (1956) records that compact bone from ox femur contains 0·1 g. of lipid/100 g. dry weight.

The present paper is a brief study of the lipids of undemineralized compact bone.

EXPERIMENTAL

Materials

Ox-femur compact-bone powder. The shafts from fresh ox femurs were freed from marrow, spongy bone, periosteum and traces of tendon at the point of attachment. The bones were washed quickly with acetone, a brush being used to clean the insides of the shafts. The latter and the points of attachment of the tendons were rasped to remove surface irregularities. A further superficial wash with acetone was given. It was considered that this procedure, whilst not removing significant amounts of compact-bone lipid, minimized contamination by bone marrow. The bones were broken into pieces measuring about 1 in. × ½ in. × ½ in. and ground to a fine powder in a Christy–Norris laboratory mill. The temperature of the powder from the mill did not rise above 30°. After mixing, one half was sieved. Any material not passing a 100 mesh British Standard sieve was reground and resieved.

A similar procedure was applied to a mixture of other ox-leg bones. This will be referred to as 'other bone material' in contrast with 'ox-femur compact bone'.

Methods

Sieving. Samples of the powders were sieved with British Standard sieves to obtain size distribution.

Moisture. Loss of weight on drying a thin layer of bone powder for 16 hr. at 105° was used to calculate moisture.

Extraction of lipid. A number of slightly different methods with Soxhlet extractors were employed on the ox-femur compact-bone powder, whereas the other bone material was directly extracted with cold acetone. Soxhlet methods: (*a*) about 50 g. of bone powder was extracted with acetone, the powder was then reground and re-extracted (the material was so fine that regrinding only amounted to remixing); (*b*) as (*a*), two thimbles being used consecutively without change of flask or solvent; (*c*) as (*b*), but with CCl_4–ethanol (9:1, v/v); (*d*) about 50 g. of bone powder was extracted with acetone, the acetone was removed from the system and the bone residue was reground and extracted with ether. Some of the acetone-soluble material in the extraction flask was insoluble in the ether and was filtered off before the lipid was isolated. The yield from 50 g. of powdered bone was 30–40 mg. of lipid.

Cholesterol. Total and free cholesterol were determined by precipitating the digitonides by a scaled-up modification of the method of Schoenheimer & Sperry (1934) as modified by Sperry & Webb (1950), and finally by weighing the digitonide. The determinations were carried out on samples of lipids isolated during the determination of total lipid and therefore suffer from the disadvantage of possible variation in purity of the isolated material. The total cholesterol in the material extracted with cold acetone was determined after hydrolysis with ethanolic 2N-KOH for 2 hr. The weight of digitonide was multiplied by 0·25 to give cholesterol, and the difference between total and free cholesterol was multiplied by 1·73 to give the weight of cholesterol esters.

Phospholipids. The Fiske & Subbarow (1925) method, as modified by Yuen & Pollard (1951) for the determination of phosphate ions, was carried out on a solution obtained by digesting the lipid extracted by method (*d*) with HNO_3 in the presence of $Ca(NO_3)_2$, evaporating to dryness and dissolving the residue in water. The lipid P was multiplied by 25 to give the weight of phospholipid.

Fatty acids. The lipid was hydrolysed with ethanolic KOH; the ethanol was evaporated off, the residue suspended in 5% NaCl (minimizing the formation of emulsions) and extracted with CCl_4 to remove unsaponifiable matter. The solution was acidified and re-extracted with CCl_4 to obtain the fatty acids, which were then isolated. Triglycerides were calculated from the fatty acid content after allowing for cholesterol esters and phospholipids.

Glycerol. This was determined on the lipid extracted with cold acetone after hydrolysis by the method of Whyte (1946) adapted for one-tenth of the quantities described. There was insufficient material for the quantitative determination of glycerol on the Soxhlet-extracted material, as less bone powder was available for analysis, but qualitative indications were obtained for its presence.

Absorption spectra. Solutions (1·01 and 0·023%, both w/v) of the cold-acetone-extracted lipid in hexane (fraction from petroleum, special for spectroscopy, British Drug

Houses Ltd.) were used for the determination of the absorption spectra in the visible and ultraviolet regions respectively.

Examination of the residue. The residue obtained from the acetone-soluble lipid after re-extraction with ether [see extraction (*d*)] was examined on a microscale. After hydrolysis, it showed strong reaction with ninhydrin and gave a value of 1·5 g. of hydroxyproline/100 g. of organic material by the Neuman & Logan (1950) technique.

RESULTS

Size distribution of powdered compact bone. The results are expressed as percentage of original material, the sieve numbers being given first. On once-ground material: not passing 22, nil; 22–36, 1%; 36–44, 4%; 44–72, 25%; 72–100, 7%; 100–150, 19%; passing 150, 44%. On reground material: not passing 100, nil; 100–150, 40%; 150–200, 22%; passing 200, 38%.

Total lipid. Protein-containing material appeared in some of the extracts. The results for the various extractions are given in Table 1, and the following observations can be made: (1) The appearance of two phases in the extracted lipid, its incomplete solubility in ether and its darker colour coincided with the extraction of increased apparent percentages of lipid from the bone. However, where these effects were very small, or were eliminated by re-extraction of the lipid, there was good agreement between the different methods. (2) The presence of protein in the lipids can be attributed to two major causes. First, when double-extraction procedures (*b*) and (*c*) are used, there is a build-up of water in the extraction system which dissolves small quantities of protein; and secondly, when the material is reground, more degraded protein is formed, which is readily soluble in traces of water. This second cause would explain the differences within group (*a*) of Table 1.

The average of the values obtained, where only traces of protein were present (eight determinations classified 'V. low' in Table 1), is 0·0673 ± 0·0035% (S.D.) of the dry ox-femur compact bone. The single value of 0·0629% obtained by the bulk extraction of compact bone from other ox-leg bones is in good agreement.

The cholesterol contents are also given in Table 1. The amounts of the cholesterol esters, calculated from the difference between total and free cholesterol, can be regarded as only approximate, as this difference is small.

The phospholipid content was 2·2% of the total lipid. The possible effect of contamination arising from the presence of inorganic phosphate is not known; however, the determination was carried out on re-extracted material. The total free fatty acids accounted for 71·5% of the Soxhlet-extracted material. The glycerol content of the cold-acetone-soluble lipid was 10·5%. From the average values obtained for free and total cholesterol contents the overall composition was calculated (Table 2).

The absorption spectrum of the lipid showed similarities to both serum β-lipoprotein (Oncley, Gurd & Melin, 1950) and pure β-carotene (Devine, Hunter & Williams, 1945). Thus a solution of the bone lipid in hexane exhibited well-defined peaks at 273 and 458 mμ, a minor peak at 430 mμ and a shoulder at 485 mμ, compared with pure β-carotene in *cyclo*hexane which possesses peaks at 275, 456 and 482 mμ and a shoulder at 430 mμ. Pure β-carotene in hexane, the complete absorption curve of which was not recorded, gave a major peak at 453 mμ (Devine *et al.* 1945).

Table 1. *Total lipid and cholesterol content of compact bone*

For methods of extraction (*a*), (*b*), (*c*) and (*d*), see Methods section.

Bone sample	Method of extraction	Total lipid (% of dry compact bone)	Protein content	Type of bone powder	Free cholesterol (% of total lipid)	Total cholesterol (% of total lipid)
Ox mixed bones	Cold acetone in bulk	0·0629	V. low	Once-ground material	10·9	12·3
Ox femur	(*a*)	0·0648, 0·0678	V. low	Once-ground material	13·3	14·3
		0·0638, 0·0697	V. low	Fraction from once-ground material passing 150-mesh	—	14·3
		0·0782, 0·0771	Low	Reground material passing 100-mesh	13·1	—
Ox femur	(*b*)	0·0987, 0·1012	High	Reground material passing 100-mesh	—	—
Ox femur	(*c*)	0·0718, 0·0802	Low	Reground material passing 100-mesh	—	—
Ox femur	(*d*)	0·0698, 0·0659 0·0737, 0·0627	V. low	Reground material passing 100-mesh	13·6	14·3

Table 2. *Composition of lipids from ox compact bone*

	Lipid extracted from ox-leg bones by cold acetone in bulk (g./100 g. of lipid)	Soxhlet-extracted lipid from ox femurs	
		(g./100 g. of lipid)	(g./100 g. of dry compact bone)
Triglycerides	85·5*	79·2	0·0535
Free cholesterol	10·9	13·3	0·0089
Cholesterol esters	2·4	1·7	0·0011
Phospholipids	Not determined	2·2	0·0015

* Not corrected for possible phospholipid content.

Serum β-lipoprotein in *cyclo*hexane shows the same general shape of absorption spectrum as the bone lipid in hexane, possessing two well-defined peaks at 270 and 455 mμ, a minor peak at 480 mμ and a shoulder at 420 mμ.

DISCUSSION

The occurrence of protein-containing matter in some of the lipids isolated from bone has been discussed in the Results section. Its association with the lipid is partly due to the technique used for extraction. Its hydroxyproline content suggests that some 10% of the protein was derived from collagen (Eastoe, 1955); the remainder was probably derived from cellular material, either as free protein or in the form of an original lipoprotein.

The dry ox femur contained 0·0673% of lipid, and the other bones 0·0629%, by extraction with cold acetone in bulk. These lipids contained 13·3 and 10·9% of free cholesterol respectively, thus giving 0·0089 and 0·0069% of free cholesterol in compact bone. If it is assumed that bone contains 20·8% of organic matter (Eastoe & Eastoe, 1954), then the organic matter contains 0·042% of free cholesterol, a value considerably higher than the 0·0028–0·0040% found by Pikular (1955) in osseocollagen. The following possibilities are suggested to account for this difference: (*a*) most of the cholesterol–protein complex postulated by Pikular is dissociated during demineralization and the bulk of the cholesterol is lost; (*b*) there are two or more sources of cholesterol, the cholesterol–protein complex being a minor one; (*c*) the cholesterol is not in fact present as a protein–cholesterol complex in the matrix but as cellular material (possibly as protein–cholesterol complexes in the cells), the small quantity remaining in the tissue after demineralization being a residue of material, the bulk of which has been removed.

Some of the triglycerides present in the lipid might arise from the presence of chylomicrons or inclusions of bone marrow.

Lindemann (1957) studied the unusual dark-blue granules present in paraffin and celloidin sections of decalcified bone and teeth stained with haematoxylin and eosin. She recalled that although these have been attributed either to calcium fluoride or to pathological calcification, she had been unable to detect calcium fluoride by an X-ray-diffraction technique. As a result of histochemical studies she suggested that the granules consisted largely of lipid material. It is possible that the lipids studied in the present work have their origin in the materials giving rise to the granules on staining. These lipids may have been derived from cellular material present in the bone.

Nikol'skaya (1953) has shown that myoalbumin, myoglobulin and myostromin from the muscles of a variety of mammals contain 0·039–0·120% of cholesterol, that found in myostromin being almost entirely in the free form. A similar state of affairs exists in compact bone, where 0·042% of the organic matter is free cholesterol and 0·005% is cholesterol esters.

SUMMARY

1. The lipid content of compact bone from ox femurs and from a mixture of other leg bones has been determined.

2. The isolated lipids have been examined and shown to contain triglycerides, cholesterol, cholesterol esters, phospholipids and probably β-carotene.

3. The free cholesterol content of compact bone has been shown to be higher than that of osseocollagen prepared from compact bone by demineralization. The reasons for this difference are discussed.

4. The relationship of the present investigation to earlier observations is examined.

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Uptake of Lead by Human Erythrocytes *in vitro*

By T. W. CLARKSON* and J. E. KENCH†
Departments of Occupational Health and Chemical Pathology, University of Manchester

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In the course of extensive studies on the effect of lead on erythrocytes *in vitro*, Aub, Fairhall, Minot & Reznikoff (1926) observed that whereas the cells became more fragile towards mechanical trauma they were more resistant to rupture by hypotonic solutions. Their experiments were made mainly on washed cells suspended in phosphate-free Ringer solutions, since the injurious effects were most manifest in such media.

It was postulated that precipitation of lead triphosphate took place on the erythrocyte membrane by reaction of lead salt with inorganic phosphate groups, with release of hydrochloric acid, and that points of localized acidity were responsible for increased fragility. Lead in whole blood was much less injurious than in phosphate-free Ringer suspensions, the difference being directly related to the inorganic phosphate content of the plasma. Any damage that was observed in cells in whole blood presumably arose by a simultaneous reaction of lead with the phosphate groups of the cell surface, which is largely dominated by such groups (Furchgott & Ponder, 1941). It follows that small quantities of lead added to whole blood should remain in the plasma as colloidal lead phosphate, whereas greater quantities would increase the fraction bound to the cells.

Certain aspects of this hypothesis have been discounted. Bischoff, Maxwell, Evans & Nuzum (1928) observed that lead salts of weak acids, e.g. lead glycerophosphate, produced changes in the erythrocyte similar to those with lead chloride, although no mineral acid was released. Bambach, Kehoe & Logan (1942) and Mortensen & Kellogg (1944) observed a marked uptake of lead by erythrocytes even at low blood levels. The latter workers observed that small quantities of the radioisotope of lead were taken up rapidly by dog erythrocytes both *in vivo* and *in vitro*, from 90% in 1 min. to a final level of 98% at the end of 15 min. The process was reversible, and markedly dependent on temperature. At concentrations greater than 1 mg. of Pb^{2+} ions/100 g. of blood, the cells may be saturated and, at the maximum level, may contain only 35% of the lead present.

The present work was undertaken to obtain more information on the interaction of lead and erythrocytes when brought into mutual contact. Our observations indicate that addition of lead salts to suspensions of erythrocytes in plasma, in isotonic sodium chloride or in Krebs–Ringer bicarbonate solutions results in the immediate formation of peptized lead phosphate sol. Combination with the cell occurs by coagulation or flocculation of the peptized sol on the cell surface.

EXPERIMENTAL

Materials

Blood from healthy young men was collected from the antecubital vein into a 0·9% NaCl solution containing 1 g. of glucose/100 ml. and anticoagulant [2 mg. of heparin B.P. (Evans Medical Supplies Ltd., Liverpool) or 3 mg. of an ammonium oxalate–potassium oxalate mixture/10 ml. of blood]. The blood was used immediately after collection or stored at 4° and used within 48 hr.

The NaCl solutions used in this work were rendered lead-free by passage through a column of sulphonated polystyrene ion-exchange resin, Zeo-Karb 225 (The Permutit Co. Ltd., London, W. 4), in the sodium cycle. The solutions in the Krebs–Ringer bicarbonate mixture (Umbreit, Burris & Stauffer, 1951) were made lead-free in a similar manner, with the ion-exchange column prepared in the appropriate cycle.

The apparatus was of Pyrex glassware which was cleaned with boiling HNO_3 and thoroughly washed with doubly

* Present address: Department of Pharmacology, Strong Memorial Hospital, University of Rochester, Rochester, New York.
† Present address: Department of Chemical Pathology, Medical School, University of Capetown.

distilled water. Where Pyrex was unobtainable soda glass was coated with silicone (Repelcote: 2% solution of a methylchlorosilane in carbon tetrachloride; Hopkin and Williams Ltd.) and cleaned with HNO_3.

Methods

Determination of lead. The organic constituents in the blood specimen were removed by a wet-oxidation technique. The blood was added to a 100 ml. Erlenmeyer flask containing 1 ml. of H_2SO_4 (sp.gr. 1·84), 2 ml. of HNO_3 (sp.gr. 1·42) and 0·5 ml. of $HClO_4$ (70%, w/v). These reagents were obtained free from lead. The mixture was maintained at 100° until the solid constituents were dispersed and a pale-yellow solution was obtained. The temperature was carefully raised until white fumes appeared. After cooling, 1 ml. of HNO_3 was added, the mixture diluted with approx. 40 ml. of doubly-distilled water and heated until H_2SO_4 alone remained. This procedure was found sufficient to remove all organic substances in quantities of blood up to 5 ml. Unless this method was carefully followed, frothing and 'bumping' of the oxidation mixture occurred, which caused extensive loss of material from the flask.

Analysis for lead was then carried out by the dithizone 'reversion technique' of Irving & Butler (1953). Two precautions, in addition to those stated by the authors, were found necessary. First, it was found that the A.R. CCl_4 solvent contained a substance which destroyed dithizone and which could not be removed by distillation. This interfering substance could be removed, however, by washing the CCl_4 with 1% neutralized hydroxylamine solution. Secondly, as a further and necessary insurance against any destruction of dithizone, 0·5 m. of 20% neutralized hydroxylamine was added to the second ammoniacal-citrate extracting solution.

Precipitation of lead from various solutions. Lead chloride (5 μg. of Pb^{2+} ions) in 0·1 ml. of 0·16 M-NaCl was added to 3 ml. of each of the following solutions maintained at 20° in Pyrex test tubes: plasma; plasma dialysed for 24 hr. against 3 l. of Krebs–Ringer bicarbonate solution; 0·16 M-NaCl; 0·16 M-NaCl exposed for 1 hr. to thrice-washed erythrocytes; Krebs–Ringer bicarbonate solution; Krebs–Ringer bicarbonate solution exposed to thrice-washed erythrocytes for 1 hr.; Krebs–Ringer bicarbonate solution containing arginine, lysine, glutamic acid, histidine, glycine, cystine, glutamine, serine, threonine, glucose, citric acid, α-oxoglutaric acid, lactic acid and urea, in concentrations equivalent to those quoted for plasma (Spector, 1956) and, in addition, 1 mg. of ammonium oxalate–potassium oxalate mixture and approx. 1 mg. of heparin (Evans Medical Supplies Ltd.). After allowing the solutions to stand for 20 min. after addition of lead, they were centrifuged at 4500 *g* in a MSE (Measuring and Scientific Equipment Co., Ltd.) medium centrifuge for 3 min., 2 ml. of the supernatant was removed and this portion and that remaining were analysed for lead. The rate of precipitation of lead added to Krebs–Ringer bicarbonate solution was followed. Lead (5 μg. as $PbCl_2$ in 0·1 ml. of 0·16 M-NaCl) was added to a series of tubes containing 3 ml. of Krebs–Ringer bicarbonate solution at 20°. After standing for various times and centrifuging at 4500 *g* for 3 min., 2 ml. of the supernatant was carefully removed in a graduated pipette and lead estimated in the sample and in the residue. Values were corrected to give the lead in 3·1 ml. of solution, which, subtracted from the total amount of lead found, provided a measure of the amount of lead precipitated.

Removal of lead by non-specific adsorbents. Weighed quantities of alumina (γ Al_2O_3 for chromatographic-adsorption analysis, British Drug Houses Ltd., London), fuller's earth (British Drug Houses Ltd., for adsorption purposes) and charcoal (British Drug Houses Ltd., activated decolorizing powder) were washed by repeated suspension in doubly-distilled water in glass-stoppered 10 ml. Pyrex test tubes. Plasma (3 ml.) was added to each, the mixture shaken for 20 min. and the plasma adjusted to pH 7·3 with dilute HCl. Lead (5 μg. as $PbCl_2$ in 0·1 ml. of 0·16 M-NaCl) was added, the mixture shaken for 20 min. at 20°, centrifuged and the plasma removed for lead analysis. Next, 3 ml. of plasma was exposed to each of the adsorbents as described and 5 μg. of lead was added after removal of the adsorbents. The plasma was allowed to stand for 20 min. at 20°, then centrifuged and 2 ml. of clear supernatant was removed for lead analysis. The residue was likewise analysed.

Uptake of lead by human erythrocytes in the presence of other metal salts and organic bases. Solutions of various cations and organic bases were prepared in 0·16 M-NaCl containing 0·01 M-barbiturate buffer, pH 6·8. At this pH the uranyl ion [$(UO_2)^{2+}$] is unstable and hence the quantity of free $(UO_2)^{2+}$ ions is very much less than that calculated from the weight of uranium present.

Buffered metal-ion solution (3 ml.) was added to 2 ml. of thrice-washed, packed erythrocytes and the suspension brought to 20° in a Warburg bath. Lead (4 μg.) was added as $PbCl_2$ in 0·1 ml. of 0·16 M-NaCl and the system allowed to equilibrate for 15 min. at 20°. After centrifuging at 4500 *g* for 3 min. the supernatant and cells were separated and their lead content was determined.

Uptake of lead by human erythrocytes in suspensions buffered at various pH *values.* Buffered solutions were prepared in the range pH 5–6 with 0·1 M-acetate buffer and in the range pH 6·0–6·6 with 0·02 M-citric acid–sodium phosphate buffer.

Thrice-washed, packed erythrocytes (2 ml.) from fresh heparinized blood were suspended in 3 ml. of each of the buffer solutions contained in 10 ml. glass-stoppered Pyrex test tubes. Below pH 6·4, addition of erythrocytes to the buffer caused a gradual raising of pH. Repeated washing with the buffer solution was necessary before a constant pH was obtained. The tubes were then immersed in a water bath at 20° and allowed to stand for 1 hr. Lead (4 μg.) was added as $PbCl_2$ in 0·1 ml. of 0·16 M-NaCl and the tubes were gently agitated in the water bath for a further 20 min. After centrifuging at 4500 *g* for 3 min. the supernatant and centrifugate were separated and their lead content was estimated.

Effect of chelating agents on uptake of lead. The chelating agents chosen for study were the disodium salt of ethylenediaminetetra-acetic acid (EDTA), sodium hexametaphosphate and glutathione, whose active lead-binding groups correspond to carboxylate, phosphate and sulphydryl respectively. A mM-solution of each reagent was prepared in 0·1 M-barbiturate buffer, pH 6·8, and the molarity of the solution adjusted to 0·16 M with NaCl.

In the first experiment, 2 ml. of thrice-washed, packed erythrocytes was suspended in 3 ml. of solution and allowed to stand for 1 hr. at 20°. Lead (4 μg.) as $PbCl_2$ was

then added and the system allowed to equilibrate in a Warburg bath at 20° for 15 min. After centrifuging at 4500 *g* for 3 min. the supernatant and cells were separated and the lead content was measured.

In the second experiment, a saline suspension containing the equivalent of 2 ml. of packed erythrocytes in 2 ml. of 0·16 M-NaCl was allowed to stand for 1 hr. at 20°. Lead (4 μg.) was then added, the system allowed to equilibrate for 15 min. and, after centrifuging at 4500 *g* for 3 min., the supernatant was removed by pipette. The centrifuged cells were resuspended in 3 ml. of chelating agent solution and allowed to stand for 15 min. at 20°. The lead content was then estimated in the cells and saline by the usual procedure.

Dialysis studies. Lead (10 μg.) as $PbCl_2$ in 0·2 ml. of 0·16 M-NaCl was added separately to two suspensions, each 6 ml., of whole oxalated blood. The suspensions were allowed to equilibrate at 20° for 0·5 hr. or for 18 hr. before dialysis against 1 l. of Krebs–Ringer bicarbonate solution containing mM-EDTA for 24 hr. The dialysis was carried out with Visking cellophan sacs, of diameter 3·8 cm., which had previously been washed free of glycerol and other impurities. After completion of dialysis the contents of the sacs were removed, their volumes measured and lead was estimated in the cells and plasma after separation by centrifuging at 4500 *g* for 3 min.

Kinetic studies of uptake of lead by human erythrocytes. A series of experiments was carried out to obtain information on the kinetics of uptake of lead by erythrocytes when suspended variously in plasma, Krebs–Ringer bicarbonate solution and NaCl solution in the presence of heparin or oxalate as anticoagulant.

Pyrex test tubes (10 ml.) containing samples of fresh heparinized blood (5 ml.) were attached by their ground-glass joints to the holders of a Warburg apparatus and immersed in the water bath maintained at 20±0·05°. After equilibration of temperature, 0·1 ml. of 0·16 M-NaCl containing 4 μg. of lead as $PbCl_2$ was added, the contents of the tube were mixed and the time was noted. The tube was allowed to stand in the bath for a measured time before centrifuging at 4500 *g* for 3 min. The uptake of lead by the cells in the blood was presumed to have ceased 30 sec. after the start of the centrifuge. After centrifuging, the supernatant plasma was removed by graduated pipette, the volume noted and the plasma transferred to a 100 ml. Erlenmeyer flask for determination of lead. Similarly, the lead content of the residual cells was determined after removal from the tube with doubly-distilled water.

Kinetic studies were made at 10° and 20° with heparinized whole blood, at 20° with oxalated whole blood and finally at 20° with 3 ml. of oxalated plasma containing the equivalent of 0·3 ml. of packed erythrocytes. The rate of uptake of lead at 37° was very rapid and precluded accurate study at that temperature.

Suspensions of erythrocytes in NaCl solution were prepared. A volume (2 ml.) from well below the top of a column of packed cells, obtained by centrifuging whole heparinized blood, was transferred to a 10 ml. glass-stoppered Pyrex test tube and washed three times by suspension in 0·16 M-NaCl. A volume (3 ml.) of 0·16 M-NaCl was then added and the tubes were allowed to equilibrate for at least 1 hr. in a Warburg water bath at 20°. This allowed the diffusible substances from the erythrocytes to reach equilibrium concentrations in the saline. Exactly the same procedure was then followed as for whole blood. A similar kinetic run was carried out at 20° for a suspension of 0·5 ml. of packed erythrocytes in 5 ml. of NaCl solution. A kinetic study with identical procedure as for NaCl solution was made on a suspension containing the equivalent of 0·3 ml. of packed erythrocytes in 3 ml. of Krebs–Ringer bicarbonate solution.

RESULTS

The reversion analytical procedure was found to give excellent linearity over a range of amounts of lead from 0 to 7 μg. Recoveries of lead added to whole blood were obtained by analysis of lead in the plasma and erythrocytes separated by centrifuging at 4500 *g* for 3 min. From timed trials it was judged that the erythrocytes were deposited and thereby removed from contact with lead 0·5 min. after the start of the centrifuge. In the range studied, the recoveries of lead (Table 1) were excellent, and demonstrated a marked predilection of lead towards the cells. The standard deviation of the values is 1%, which as applied to a 5 μg. quantity of lead is equivalent to a variation of ±0·05 μg.

There was a possibility that some of the added lead might have formed a precipitate with inorganic phosphate in the plasma (invisible owing to the minute amounts of lead used) and was carried down with the cells on centrifuging and consequently included in values for uptake of lead by the cells. A similar result could arise with several of the other suspending media, many of

Table 1. *Recoveries of added lead from 5 ml. of whole blood calculated from analyses of plasma and cells after separation by centrifuging*

Lead added (μg.)	Lead found (μg.)			Added lead recovered (μg.)	Recovery (%)
	Plasma	Cells	Total		
0·00	0·08	1·67	1·75	—	—
0·63	0·12	2·27	2·39	0·64	101
1·25	0·14	2·85	2·99	1·24	99
1·87	0·13	3·48	3·61	1·86	100
2·50	0·15	4·11	4·26	2·51	100
3·12	0·14	4·79	4·93	3·18	102
4·38	0·14	6·01	6·15	4·40	100

Table 2. *Deposition of lead from various media*

After the addition of 0·1 ml. of 0·16 M-NaCl containing the equivalent of 5 μg. of lead the system was allowed to stand for 20 min. at 20°. Precipitated lead was removed by centrifuging at 4500 *g* for 3 min.

	Suspension medium					
	Oxalated plasma	0·16 M-NaCl	0·16 M-NaCl exposed to erythrocytes	Krebs–Ringer bicarbonate soln.	Krebs–Ringer bicarbonate soln. after exposure to erythrocytes	Krebs–Ringer bicarbonate soln. containing heparin, oxalate and small organic molecules
pH of medium after addition of $PbCl_2$	7·31	5·50	6·80	7·39	7·39	7·39
Percentage of added lead precipitated	7·3	0·0	10·0	84	20	84

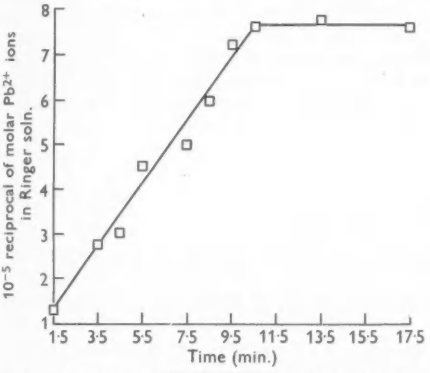

Fig. 1. Precipitation of lead from Krebs–Ringer bicarbonate solution at pH 7·39 and 20°. Results are plotted as a test for second-order kinetics with respect to lead.

Table 3. *Removal of added lead from plasma by non-specific adsorbents or from plasma after exposure to the same adsorbents*

For experimental details see text.

	% of added lead removed by centrifuging	
Adsorbent used	Suspension of adsorbent in plasma	Plasma after exposure to adsorbent
Chromatographic alumina (2 g.)	97	7·1
Activated charcoal (2 g.)	98	6·8
Fuller's earth (2 g.)	96	7·0

which contained inorganic phosphate at pH 6·8–7·4. In Krebs–Ringer bicarbonate solution (Table 2) 84% of the lead added was precipitated in this manner. The rate of precipitation was measured (Fig. 1) and plotted as a test for second-order kinetics, which apply to the uptake of lead by erythrocytes. A fairly close agreement with second-order kinetics was apparent, but it was difficult to assess the exact value of the time of cessation of the precipitation since this would continue during centrifuging. Deposition of already-formed precipitate was assessed to require 1·5 min., and the precipitate measured after centrifuging for 3 min. was deemed to represent that formed until 1·5 min. after start of the centrifuge.

After exposure of the Krebs–Ringer bicarbonate solution to erythrocytes much less lead was removed, and in plasma, the ionic content of which was similar to that of Krebs–Ringer bicarbonate solution, 7·3% only of the added lead was precipitated. Lead added was, however, readily removed by non-specific adsorbents (Table 3). Moreover, the plasma after exposure to such adsorbents still retained its property of stabilizing lead, confirming that the non-specific adsorbent was directly responsible for removal of the heavy metal.

No interference with attachment of lead to human erythrocytes in buffered NaCl suspension is brought about by any of a number of other metal salts and organic bases [K^+, Ca^{2+} ions up to 0·16 M; Mg^{2+} ions, acetylcholine chloride, methylene blue, quinine, up to 10 mM; Cu^{2+}, Hg^{2+}, $(UO_2)^{2+}$, Zn^{2+}, Al^{3+}, $(Co[NH_3]_5,H_2O)^{3+}$, Te^{4+} ions up to mM]. With an initial lead concentration (immediately after addition of lead chloride) of 7 μM, all competing species were present in great excess, having molar concentrations of 150–10 000 times that of lead. The uptake of lead was very much inhibited by an increased H^+ ion concentration in the medium (Fig. 2): a pH change from 6·6 to 6·0 had a profound effect.

It is clear that chelating agents such as EDTA and sodium hexametaphosphate largely prevent the passage of lead on to the erythrocytes, but lead already attached can be withdrawn only very

slowly even by powerful chelating agents of the metal (Table 4).

Dialysis studies (Table 5) confirmed that added lead was removable from the cells after contact with lead for 18 hr. Nevertheless, the lead of the cells as obtained from the donor could not be removed by dialysis against EDTA.

The data collected on the kinetics of uptake of lead by erythrocytes are presented in Figs. 3–6. Removal of lead by the cells, as computed from concentration in the suspending media, agreed well with second-order criteria in all the experiments, except when the medium was Krebs–Ringer bicarbonate solution (Fig. 4). The specific velocity constant was very much influenced by the composition of the suspending solution. Thus for whole heparinized blood $k_{20°} = 5{\cdot}61 \times 10^4$ min.$^{-1}$ (Fig. 5), and for a 0·9% NaCl suspension of cells from the same blood specimen $k_{20°} = 29{\cdot}0 \times 10^4$ min.$^{-1}$ (Fig. 3). It is also apparent that the reaction velocity was approximately proportional to the total volume of cells present both in saline and in whole blood. In saline (Fig. 3) the reaction rates are 29×10^4 min.$^{-1}$ and $7{\cdot}8 \times 10^4$ min.$^{-1}$, a ratio of 3·7, corresponding to a cell volume ratio of 4·0. In oxalated blood (Figs. 5 and 6) the specific-reaction rates, $k_{20°}$, are $4{\cdot}5 \times 10^4$ and $0{\cdot}55 \times 10^4$ respectively, giving a ratio 8·2 similar to the cell volume ratio 7·8. This relationship between velocity constant and cell volume did not hold closely when oxalated blood was compared with heparinized blood (Fig. 5), the ratio of the constants being 1·25 as compared with a cell volume ratio of 0·91. The cells were from different specimens of blood, and this may explain the apparent anomaly. The two linear curves in Fig. 5 demonstrate that the velocity of uptake of lead was dependent on temperature:

$$k_{20°}/k_{10°} = 5{\cdot}61 \times 10^4/2{\cdot}16 \times 10^4,$$

giving Q_{10} of 2·6 and an activation energy of 15 800 cal. mole^{-1}.

Erythrocytes (0·3 ml.) in whole oxalated blood treated with 5 μg. of lead at 20° (Fig. 6) reached a saturation level in about 60 min., equivalent to 11·4 μg. of lead/ml. of packed cells, which compares

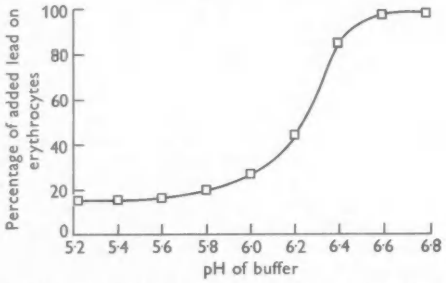

Fig. 2. Uptake of lead by human erythrocytes in buffered-saline suspension, as a function of pH. Packed erythrocytes (2 ml.) were suspended in 3 ml. of saline buffer solution and were allowed to stand for 20 min. after the addition of 5 μg. of lead.

Table 4. *Distribution of* Pb^{2+} *ions between human erythrocytes and chelating agents*

Expt. 1: packed cells (2 ml.) were added to 3 ml. of 0·16 M-NaCl containing the chelating agent, final concentration mM. Pb^{2+} ions (4 μg.) were added and the suspension was allowed to stand at 20° for 15 min. Expt. 2: 4 μg. of Pb^{2+} ions were allowed to equilibrate for 15 min. with 2 ml. of packed cells suspended in 5 ml. of NaCl soln. The leaded cells were separated and suspended for 15 min. in 3 ml. of 0·16 M-NaCl containing the chelating agent, concentration mM.

	Expt. 1		Expt. 2	
Chelating agent	% of added Pb^{2+} ions in cells	% of added Pb^{2+} ions in chelate solution	% of added Pb^{2+} ions in cells	% of added Pb^{2+} ions in chelate solution
Ethylenediaminetetra-acetic acid	9	91	74	26
Sodium hexametaphosphate	8	92	91	9
Glutathione	48	52	95	5

Table 5. *Removal of lead from erythrocytes by dialysis*

Whole oxalated blood (6 ml.) containing the equivalent of 3 ml. of packed erythrocytes was equilibrated at 20° for different times with 8·33 μg. of lead as $PbCl_2$. The blood specimen was contained in a Visking cellophan sac and dialysed for 24 hr. against 1 l. of Krebs–Ringer bicarbonate solution containing mM-ethylenediaminetetra-acetic acid.

		Lead found before dialysis (μg.)				Lead found after dialysis (μg.)	
Time of equilibration of lead with whole blood (hr.)	Quantity of lead added (μg.)	Plasma	Cells	Total	Pb found in excess of quantity added	Plasma	Cells
0·5	8·33	0·72	9·71	10·43	2·10	0·45	2·26
18·0	8·33	0·38	10·22	10·60	2·27	0·00	2·21

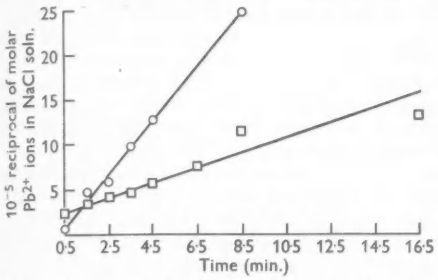

Fig. 3. Uptake of lead by human erythrocytes in 5 ml. of NaCl suspension at 20° containing the equivalent of 0·5 (□) or 2 (○) ml. of packed erythrocytes.

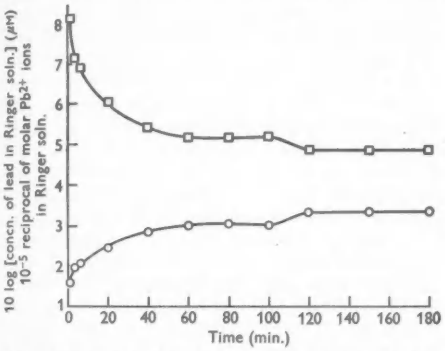

Fig. 4. Uptake of lead by human erythrocytes, equivalent to 0·3 ml. of packed cells suspended in 3 ml. of Krebs–Ringer bicarbonate solution at 20°. Results are plotted for 10 log (Pb^{2+} ions in Ringer) μM (□) and for 10^{-5} ($1/Pb^{2+}$ ions in Ringer) (M^{-1}) (○).

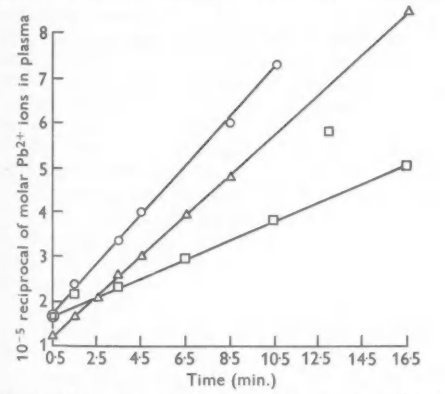

Fig. 5. Uptake of lead by human erythrocytes in oxalated whole blood at 20° (△) and in whole heparinized blood at 10° (□) and 20° (○). Results are plotted as a test for second-order kinetics with respect to lead.

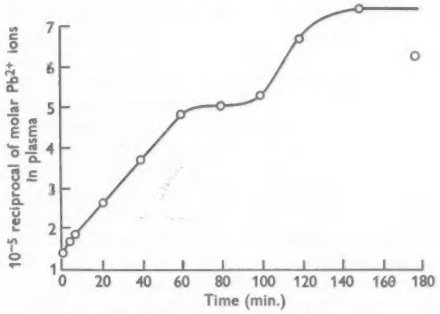

Fig. 6. Uptake of lead by human erythrocytes in whole oxalated blood at 20°, containing the equivalent of 0·3 ml. of packed erythrocytes in 3 ml. of plasma. Results are plotted for 10^{-5} (reciprocal of the molar concentration of lead in plasma).

well with the value quoted by Bambach *et al.* (1942) of 0·55 mg. of lead/100 g. of blood. After approx. 90 min. a further smaller uptake of lead occurred. A similar situation arose when 0·3 ml. of cells was suspended in Krebs–Ringer bicarbonate solution (Fig. 4). Also the two curves exhibit saturation phenomena after about 60 min. With the Krebs–Ringer bicarbonate suspension, however, the experimental values do not obey second-order criteria and much less lead is taken up by the cells.

DISCUSSION

In agreement with the findings of Bambach *et al.* (1942) and Mortensen & Kellogg (1944) the present investigations have shown that when 5 μg. of lead as lead chloride is added to 5 ml. of whole blood, over 95% is rapidly attached to the cells. It is difficult to see how such a distribution of lead could occur if the metal was taking part in the reaction as the bivalent cation Pb^{2+}, since plasma components such as glycerophosphate, phospholipids and proteins contain groups such as phosphate, sulphydryl and carboxyl, all of which can bind ionic lead and thereby prevent its uptake by the cell. Likewise if attachment to the cell involved reaction of Pb^{2+} ions with such groups on the cell membrane, competitive inhibition of uptake of lead would be expected from cations of similar properties. Yet Table 4 demonstrates that none of a wide variety of potential competitors tested showed a significant effect. Clearly lead must be operative in some other form or combination, as a metal complex or chelate with organic plasma components or as a dispersed colloidal metal salt.

Aub *et al.* (1926) emphasized the importance of inorganic phosphate for the stability of lead salts in whole blood and saline suspensions. The work of Millet & Jowett (1929) and Maxwell & Bischoff

(1929) demonstrated that under the conditions of ionic strength and pH of plasma, lead triphosphate would tend to be precipitated when the concentration of lead was raised to levels above approximately 0·1 μM. Lead present in plasma after equilibration with cells is of a concentration of about the same order, namely 0·5 μM. Goldberg, Ashenbrucker, Cartwright & Wintrobe (1956) have observed that the inhibition of haem synthesis *in vitro* by lead in this range of concentration was less in phosphate buffer than in 2-amino-2-hydroxymethylpropane-1:3-diol buffer. Table 2 records the quantity of lead precipitated when amounts of lead equivalent to a concentration of about 5 μM are added to various solutions. Almost complete precipitation of lead, presumably as the triphosphate salt, occurred in Krebs–Ringer bicarbonate solution, which has an inorganic phosphate concentration identical with the value quoted for serum (Power, 1953). Yet in plasma, 0·16 M-sodium chloride at pH 6·8 or Krebs–Ringer bicarbonate solution exposed to erythrocytes for 1 hr., very little precipitation of lead was observed. It seems probable that the addition of lead salts to these solutions will result first in the formation of lead triphosphate. Normally this would coagulate or flocculate until precipitation occurred. But the second stage is prevented by a 'peptization' process whereby organic substances in the plasma, or originating from the cell (Lovelock, 1954), are adsorbed on the surface of the particles of the lead phosphate sol and prevent further coagulation, a common phenomenon when insoluble metal-salt sols are formed in the presence of such substances as agar, gelatin or citric acid (Packter & Matalon, 1954). Such metal-salt sols, other than at their equivalence point, carry electrical charges and may be expected to be removed from the suspending media by non-specific adsorbents. Such was the case for lead in plasma (Table 3). The removal of lead from plasma by cells might be a similar adsorption mechanism.

The importance of the sol form of lead phosphate was demonstrated by the effect of pH on the cell interaction (Fig. 2). As the pH of the supporting medium fell from 6·6 to 6·0, uptake of lead by the cells was markedly reduced. It is in this range that the highly insoluble lead triphosphate is transformed into the more soluble lead monohydrogen phosphate, whereby the sol nature of the lead phosphate would be destroyed. The charge on the lead triphosphate sol is determined by the ratio of Pb^{2+} ions to PO_4^{3-} ions in the medium. In the presence of excess of phosphate, as in plasma and Krebs–Ringer bicarbonate solution, the sol would bear a negative charge. In saline suspensions of thrice-washed erythrocytes, the concentration of free phosphate would be very low so that the net charge on the lead sol could well be reversed. This would account for the more rapid reaction with the negatively charged erythrocyte in saline as compared with Krebs–Ringer bicarbonate solution or plasma (Figs. 3–5). These results imply that the protective action of plasma and of other solutions containing excess of phosphate is due, not to competition for available Pb^{2+} ions, as proposed by Aub and his colleagues, but to the fact that the approaching lead phosphate sol carries a negative charge in such a medium. Changes in sedimentation rate and fragility of the cells have been discussed elsewhere in terms of this concept (Clarkson & Kench, 1958).

The kinetic studies of uptake of lead by human erythrocytes (Figs. 3–6) emphasize, however, that the process is not a simple adsorption of the phosphate sol on the cell surface. The reaction is markedly influenced by temperature, having a Q_{10} of 2·8 and an activation energy of 15 800 cal. mole^{-1}. This is typical of a chemical reaction rather than an adsorptive process. Mortensen & Kellogg (1944) first noted the influence of temperature on uptake of lead by erythrocytes and arrived at a similar conclusion. All the kinetic data closely obey second-order reactive criteria for the lead taking part, which again discounts a simple adsorption process. The values obtained by Mortensen & Kellogg for uptake of lead by dog erythrocytes and plotted for first-order kinetics do not obey linearity at 20°. If these values are plotted for second-order requirements, excellent linearity is obtained. An examination of the curve in Fig. 6, for cells present in only small quantity, reveals that the cells do not enter directly into the reaction with lead. Even at points very close to the cessation of uptake of lead, second-order characteristics are still obeyed. Such could arise only from the collision of two lead atoms with the cell surface always in excess and not a determinant of the observed kinetics. Nevertheless, the cells markedly affect the reaction rate and do so in a regular fashion, as outlined in the Results section. It seems likely that such second-order kinetics refer to coagulation of the peptized lead sol (Packter & Matalon, 1954), with the cell surface acting as a catalyst to the reaction; in other words, uptake of lead occurs by the combination of the peptized lead phosphate sol particles on the surface of the cell to form a precipitate or larger particulate form of lead phosphate. The cell surface, being largely dominated by phosphate groups, would provide an ideal surface for such an aggregation. Once the coagulation of lead phosphate sol on the cell surface has been completed, the lead salt will have properties similar to those of the usual precipitate, namely that the reverse reaction of dispersion of the coagulate is slow. This is borne out in Table 4. Although chelating agents such as EDTA, hexametaphosphate and glutathione will bind ionic lead quite strongly and

effectively prevent uptake by the cell, they remove the metal salt only very slowly once it is attached to the cell. Lead phosphate added to cells could, however, be removed by prolonged dialysis against Krebs–Ringer bicarbonate solution containing mM-EDTA (Table 5). Since Foreman & Trujillo (1954) have shown that EDTA cannot enter the erythrocyte, it is clear that the lead removed by the chelating agent must be on the surface of the cell and remain there for at least 24 hr. after the initial uptake. Lead originally present in the cells when the blood specimen was collected could not be removed by this treatment and was presumably located intracellularly. Such intracellular lead, responsible during maturation of the erythrocyte for inhibition of several stages in biosynthesis of haem, is probably in a form other than phosphate, since a peptized phosphate sol would hardly provide sufficient Pb^{2+} ions for inactivation of enzyme.

SUMMARY

1. A study is described of the uptake of lead *in vitro* by human erythrocytes, including experiments on the form of lead in plasma and the kinetics of lead-binding.

2. Lead appears to exist in plasma as a peptized lead phosphate sol, which is rapidly aggregated in a second-order reaction to particulate form on the surface of the erythrocytes. More than 95% of small quantities of added lead are rapidly attached to the cells.

3. No competitive inhibition of uptake of lead was observed with any of a number of potential competitors tested, including other heavy metals, Cu^{2+}, Hg^{2+}, $(UO_2)^{2+}$ and Tl^{4+} ions.

4. Chelating agents such as ethylenediaminetetra-acetic acid, hexametaphosphate and glutathione remove lead attached to erythrocytes only slowly *in vitro*; intracellular lead was not withdrawn.

5. It is suggested that the protective effect of plasma and of other solutions containing excess of phosphate is due to a charge effect on the lead phosphate sol, leading to additional negative charge on the erythrocyte.

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Manometric Studies of Bracken [*Pteridium aquilinum* (L.) Kuhn] Thiaminase

By R. H. KENTEN

Biochemistry Department, Rothamsted Experimental Station, Harpenden, Herts

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Bracken thiaminase catalyses the fission of the methylene–quaternary nitrogen bond of thiamine with transfer of the pyrimidine moiety to an amine acceptor (see, for example, Fujita, Okamoto & Nose, 1955; Kenten, 1957) according to the general equation

$$P\text{–}CH_2\text{–}T^+ + R\text{–}NH_2 \rightarrow P\text{–}CH_2\text{–}NHR + T + H^+, \quad (1)$$

where P and T stand for the pyrimidine and thiazole components of thiamine. Therefore it should be possible to study the transfer reaction of thiaminase manometrically in bicarbonate–carbon dioxide buffer by measuring the carbon dioxide output which would accompany the release of the H^+ ion. In the present work the suitability of the manometric method for studying the action of bracken thiaminase has been examined.

MATERIALS AND METHODS

Plant material and extracts. Bracken fronds were harvested locally in July and August. The pinnae were stripped from the rachis and passed through a domestic meat mincer. The mince was mixed with 1·5 vol. of 0·033 M-Na_2HPO_4 and squeezed by hand through strong cotton cloth. Four such extracts were made with thiaminase activities of 66, 42, 20 and 28 units/ml.

Thiaminase preparations. Two preparations were made from fresh bracken. The extracts were filtered through fluted Whatman no. 1 filter papers at 0–4° for 2 days; any extract which had not passed through in this time was clarified by centrifuging at 10 000 *g* for 15 min. and the supernatant added to the filtrate. The method of Kenten (1957) was then followed as far as the second precipitation with $(NH_4)_2SO_4$. This precipitate was suspended in the minimum amount of 0·01 M-phosphate buffer, pH 7·5, and dialysed against several changes of distilled water at 0–4° for 24 hr. The first preparation made from bracken gathered in early July consisted of a dark-brown mobile fluid (enzyme *A*, 0·6 mg. of N/ml.). It contained 49 % of the thiaminase present in the extract. The specific activity was 1100, representing a tenfold purification. For the second preparation bracken was gathered in late August. Difficulty was encountered throughout the preparation owing to the presence of large amounts of mucilage. The dialysed second $(NH_4)_2SO_4$ precipitate (enzyme *B*, 1·28 mg. of N/ml.) had 35 % of the activity of the original extract and was highly viscous. Fractionation of part of enzyme *B* with calcium phosphate gel as described by Kenten (1957) gave a light-brown mobile fluid (enzyme *C*, 0·14 mg. of N/ml.) with 55 % recovery of its activity. The preparations were stored frozen at −18°, at which temperature no appreciable loss of activity was observed in two months. Apart from the differing specific activities of these preparations, no other differences of activity in the thiaminase-transfer reaction were found.

Enzyme units. One unit of thiaminase activity is that amount of enzyme which catalyses the formation of 1 μmole of *N*-(4-amino-2-methylpyrimidin-5-yl)methylpyridine ion (heteropyrithiamine) in 1 hr. from a mixture of thiamine and pyridine under the standard conditions described by Kenten (1957). The specific activity is the number of enzyme units/mg. of N of the enzyme preparation.

Estimation of thiamine and heteropyrithiamine. After deproteinization of the reaction mixtures with metaphosphoric acid, thiamine was estimated by the method of Melnick & Field (1939) and heteropyrithiamine by the method of Kenten (1957).

Nitrogen and other elements. The nitrogen content of the bracken preparations was determined by a micro-Kjeldahl method and the NH_3 measured by titration with HCl. Other analyses were by Weiler and Strauss, Oxford.

Thiamine chloride hydrochloride. This was of B.P. quality from Roche Products Ltd. Solutions were prepared and adjusted to pH 7·5 with dilute $NaHCO_3$ immediately before use.

Manometric experiments. These were carried out in the Warburg apparatus. The standard conditions referred to in the text are when the total volume of the reaction mixture was 3 ml., the gas phase $CO_2 + N_2$ (5:95, v/v), the reactants, with the exception of $NaHCO_3$, previously adjusted to pH 7·5, the $NaHCO_3$ concentration 0·05 M and the temperature 37°. Under these conditions the pH of the reaction mixtures would be 7·5.

Melting points. These are uncorrected.

Preparation of thiamine analogues

4-Amino-5-bromomethyl-2-methylpyrimidine hydrobromide was prepared by the method of Cline, Williams & Finkelstein (1937); heteropyrithiamine bromide hydrobromide by the method of Kenten (1957); *N*-(4-hydroxy-2-methylpyrimidin-5-yl)methyl-5-(β-hydroxyethyl)-4-methylthiazolium chloride hydrochloride (oxythiamine) according to the directions of Rydon (1951); and *N*-(4-amino-2-methylpyrimidin-5-yl)methylaniline by the method of Fujita, Nose, Uyeo & Koizumi (1952*b*).

N-(4-amino-2-methylpyrimidin-5-yl)methylquinolinium bromide hydrobromide (*quinilinothiamine*). 4-Amino-5-bromomethyl-2-methylpyrimidine hydrobromide (300 mg.) was dissolved in 12 ml. of *iso*propyl alcohol and 1 g. of quinoline was added. The mixture was kept at 25° for 6 days. The pale-yellow precipitate was washed with *iso*propyl alcohol and ether and dried *in vacuo* to give 252 mg., m.p. 234–8° (decomp.). Repeated recrystallization from aqueous methanol gave slightly greenish yellow crystals which, after drying *in vacuo* over conc. H_2SO_4, had m.p. 255–256° (decomp.); Matsukawa & Yurugi (1954) give 261° (decomp.) [Found: Br 37·8. Calc. for $C_{15}H_{16}N_4Br_2,\frac{1}{2}H_2O$ (Matsukawa & Yurugi found $\frac{1}{2}$ mol. of H_2O): Br 38·0 %].

5-(β-Hydroxyethyl)-4-methyl-3-(o-nitrobenzyl)thiazolium chloride. 5-(β-Hydroxyethyl)-4-methylthiazole (2 g.) and 2 g. of *o*-nitrobenzyl chloride in 2 ml. of xylene were heated at 100° for 86 hr. The brownish crystalline mass which separated was washed with xylene and ether and dried *in vacuo* to give 3·64 g., m.p. 203–205° (decomp.). Recrystallization from aqueous ethanol and drying *in vacuo* over conc. H_2SO_4 gave pale buff-coloured crystals, m.p. 204–205° (decomp.) (Found: C, 49·2; H, 5·1; Cl, 11·7. $C_{13}H_{15}O_3N_2SCl$ requires C, 49·5; H, 4·8; Cl, 11·3 %).

4-Methyl-3-(o-nitrobenzyl)thiazolium chloride. This was prepared by a method similar to that of Clarke (1944). 4-Methylthiazole (1·4 g.) and 2 g. of *o*-nitrobenzyl chloride in 2 ml. of xylene were heated at 125° for 24 hr. The crystalline mass of faintly brownish needles was washed with xylene and ether and dried *in vacuo* to give 1·84 g., m.p. 200–201° (decomp.). Recrystallization from ethanol–toluene gave faintly buff-coloured crystals which, after drying *in vacuo* over conc. H_2SO_4, had m.p. 204° (decomp.). Clarke (1944) gives 186·5–187° (decomp.) (Found: N, 10·2; Cl, 12·9. Calc. for $C_{11}H_{11}O_2N_2SCl$: N, 10·4; Cl, 13·1 %).

3-(o-Aminobenzyl)-4-methylthiazolium chloride hydrochloride. This was prepared by a method similar to that of Clarke (1944), except that the *o*-nitro compound was reduced by treatment with 3·5 moles of $SnCl_2,2H_2O$ in conc. HCl at 80–90° for 45 min. The procedure of Clarke was then followed. The white crystalline product turned orange at about 190° and then gradually darkened with further increase in temp. up to 270° without melting. Clarke records decomposition over the range 204–212° (Found: Cl, 25·6 %; equiv. by titration 281. Calc. for $C_{11}H_{14}N_2SCl_2$: Cl, 25·6 %; mol.wt. 277).

N-(4-Amino-2-methylpyrimidin-5-yl)methylpiperidine. 4-Amino-5-bromomethyl-2-methylpyrimidine hydrobromide (167 mg.) was dissolved in 2·5 ml. of absolute ethanol and 400 mg. of piperidine was added. The mixture was kept at 37° for 24 hr. and then concentrated to dryness over conc.

H_2SO_4 *in vacuo*. The residue was extracted several times with boiling ether and the combined ether extracts were evaporated to dryness. The white crystalline solid (108 mg.) of m.p. 80–84° was recrystallized from aqueous ethanol, to give colourless crystals. Drying *in vacuo* over conc. H_2SO_4 gave an opaque white solid of m.p. 97°, unchanged on further recrystallization and drying *in vacuo* (Found: C, 63·7; H, 8·8; N, 27·0. $C_{11}H_{18}N_4$ requires C, 64·0; H, 8·7; N, 27·2%).

RESULTS

Theoretical basis of the manometric method

In the thiaminase transfer reaction (equation 1) two bases react to give two other bases which may differ considerably from the reactants in the strength of their ionizable groups. The reaction takes place readily at pH 7·5 and if, at this pH, the extent of ionization of the products is less than that of the reactants, acid will be released and in bicarbonate–CO_2 buffer a CO_2 output would be expected to accompany thiaminase action. The following examples make this clear.

(*a*) If the acceptor amine and the pyrimidinylmethyl–amine product are both very weak bases (pK less than 5·5) the general equation (1) is an exact formulation of the reaction, and at pH 7·5 all the H^+ produced will enter into the equilibrium

$$H^+ + HCO_3^- \rightleftharpoons H_2CO_3 \rightleftharpoons CO_2 \text{ (dissolved)} \rightleftharpoons CO_2 \text{ (gas)} \quad (2)$$

and the evolution of 1 mole of CO_2 would be expected.

(*b*) If, however, the pyrimidinylmethyl–amine product is a strong base it will be ionized:

$$P\text{–}CH_2\text{–}T^+ + R\text{-}NH_2 \rightarrow P\text{–}CH_2\text{–}NH_2^+\text{–}R + T \quad (3)$$

and no evolution of CO_2 will be observed. It is possible therefore to have an output of between 0 and 1 mole of CO_2 when the acceptor amine is a weak base.

(*c*) With a strong base as the acceptor amine the two extreme possibilities may be written:

$$P\text{–}CH_2\text{–}T^+ + R\text{-}NH_3^+ \rightarrow P\text{–}CH_2\text{–}NH_2^+\text{–}R + T + H^+ \quad (4)$$

$$P\text{–}CH_2\text{–}T^+ + R\text{-}NH_3^+ \rightarrow P\text{–}CH_2\text{–}NHR + T + 2H^+ \quad (5)$$

and an output of between one and two moles of CO_2 would be expected, according to the extent of ionization of the pyrimidinylmethyl–amine product. At pH 7·5 the primary amino group (pK_a 4·5, Williams & Ruehle, 1935) attached to the pyrimidine heterocycle of thiamine will not be ionized, and substitution of a different base for the thiazole moiety would not be expected to lead to an increase in its strength sufficient to cause appreciable ionization at pH 7·5.

Table 1 shows how the expected CO_2 output varies with the pK_a values of the acceptor amine and of the pyrimidinylmethyl–amine product when the thiaminase reaction takes place at pH 7·5.

Total outputs of carbon dioxide with different amines

Preliminary experiments, in which a colorimetric method of estimating thiamine destruction was used, suggested that, of a number of amines which were active in the bracken thiaminase-catalysed transfer reaction, pyridine, aniline, piperidine and trimethylamine were among the most active. These amines, either on account of their difference in ionization at pH 7·5, or because of the degree of ionization of the product they give by the thiaminase reaction, are suitable for testing experimentally a number of the examples discussed above.

The experiments were made under the standard conditions, with 0·3 ml. of enzyme *A* and a concentration of amine of 0·05 M. The results are given in Table 2 and Fig. 1. Corrections for CO_2 retention were estimated by adding known amounts of standard acid either to the reaction mixtures at the end of the experiment or to mixtures made up to correspond closely in composition to these final reaction mixtures. The amount of bound CO_2 was found to be small and varied from 7·5 to 11% of the experimental output.

Pyridine. Pyridine is a weak base (pK_a 5·1 at 37°) and the transfer reaction leads to the formation of heteropyrithiamine, which has the quaternary-nitrogen configuration and is a strong base. Therefore the reaction proceeds according to equation (3) and no evolution of CO_2 would be

Table 1. *Relation between the expected output of carbon dioxide and the ionization constants of the acceptor amine and the pyrimidinylmethyl–amine product*

The results are given as moles of CO_2/mole of thiamine destroyed at pH 7·5.

pK_a of acceptor amine	pK_a of pyrimidinylmethyl–amine product					
	4–5	6	7	8	9	10–11
4–5	1·0	0·97	0·75	0·25	0·03	0
6	1·03	1·0	0·78	0·28	0·06	0·03
7	1·25	1·22	1·0	0·50	0·28	0·25
8	1·75	1·72	1·50	1·0	0·78	0·75
9	1·97	1·94	1·72	1·22	1·0	0·97
10–11	2·0	1·97	1·75	1·25	1·03	1·0

expected. Experimentally no CO_2 output was found in a period of 1 hr., although estimation of heteropyrithiamine in the reaction mixtures showed that 14·1 μmoles had been formed from the 14·8 μmoles of thiamine added. No thiamine was detected in these mixtures at the end of the reaction.

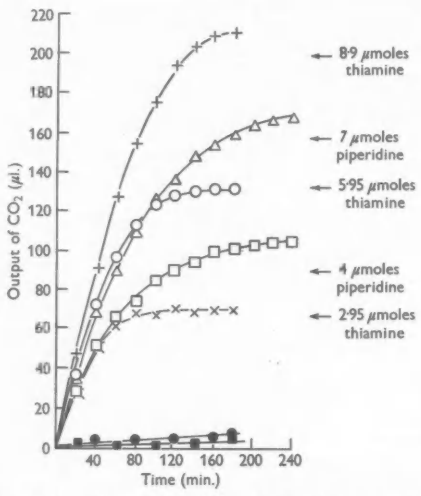

Fig. 1. Dependence of the CO_2 output on the amounts of thiamine and piperidine present. The experiments were made under the standard conditions. When thiamine was varied, the reaction mixtures contained 0·05 M-piperidine and 0·3 ml. of enzyme *A* and 2·95 (×), 5·95 (○), or 8·9 (+) μmoles of thiamine. When piperidine was varied, 0·5 ml. enzyme was used, the thiamine concn. was 0·005 M and the mixtures contained 4 (□) or 7 (△) μmoles of piperidine. ■, Enzyme heated for 15 min. at 100° before use, 8·9 μmoles of thiamine and 0·05 M-piperidine, ●, 8·9 μmoles of thiamine, piperidine omitted. The values are corrected for the blank outputs when thiamine was omitted, but not for CO_2 retention. The arrows show the outputs in terms of 1 mole of CO_2/mole of the limiting reactant.

Aniline. Titration of synthetic *N*-(4-amino-2-methylpyrimidin-5-yl)methylaniline (the expected product) with dilute hydrochloric acid at the glass electrode gave pH 6·6 as the mid-point of the neutralization curve of the aniline nitrogen group at 22°. By applying the temperature correction of Hall & Sprinkle (1932) the pK_a of the aniline nitrogen group at 37° would be about 6·3 and at pH 7·5 it would be partly ionized. Since aniline is a weak base (pK_a 4·4 at 37°) an output of 0·94 mole of CO_2 would be expected from the thiaminase reaction, according to equations (1) and (3). Experimentally a mean value of 0·89 mole of CO_2/mole of thiamine destroyed was obtained.

Piperidine. Piperidine is a moderately strong base (pK_a 10·8 at 37°) and it would be fully ionized at pH 7·5. The pK_a of the piperidine nitrogen group of the pyrimidinylmethylpiperidine product was estimated in the same way as for the aniline compound and found to be about 8 at 37°. The thiaminase reaction would therefore be expected to lead to the evolution of 1·25 moles of CO_2/mole of thiamine destroyed, according to equations (4) and (5) and Table 1. A mean value of 1·15 moles of CO_2 was obtained experimentally. It can be seen from the general equation (1) that either the amount of thiamine or the amount of amine present can limit the reaction. It was found that when thiamine was present in excess the CO_2 outputs obtained were slightly greater, the mean value being 1·25 moles of CO_2/mole of piperidine added.

Trimethylamine. Trimethylamine (pK 9·5 at 37°) would be 99 % ionized at pH 7·5; since the product of the thiaminase reaction would have the quaternary-nitrogen configuration, a CO_2 evolution of 0·99 mole/mole of thiamine destroyed would be expected, from equations (3) and (4). With thiamine

Table 2. *Total outputs of carbon dioxide with different amines*

The experimental conditions were similar to those of Fig. 1 except that either enzyme *A* or *C* was used. When the amine was present in excess, 2·95, 5·95 or 8·9 μmoles of thiamine were added. When thiamine was present in excess its concentration was 0·005 M and 4 or 7 μmoles of piperidine were added. The values are corrected for CO_2 retention. Blank uptakes obtained when thiamine was omitted have been subtracted. The evolution of CO_2 had stopped in all experiments after 4–5 hr.

Amine present	Amine concn. (M)	Total output of CO_2 (moles/mole of thiamine destroyed) Expected	Found
Pyridine	0·05	0	0
Aniline	0·05	0·94	0·89
Trimethylamine	0·05 or 0·1	0·99	0·90
Piperidine	0·05	1·25	1·15
(Piperidine; thiamine in excess)	—	(1·25)*	(1·25)*

* Moles of CO_2/mole of piperidine added.

as limiting substrate the mean value found experimentally was 0·90 mole of CO_2. Experiments with trimethylamine in limiting amount were not attempted because of the rapid decrease in reaction velocity with decreasing trimethylamine concentration (Table 4).

The results show that with piperidine, aniline and trimethylamine rather less than the expected CO_2 output was obtained. The pK_a values for the products used for calculating the CO_2 outputs when piperidine and aniline are the acceptors are only approximate and may account for part of the discrepancy. With trimethylamine there is no such uncertainty; yet, when thiamine was limiting, low values for the total CO_2 output were also obtained. Such results could be accounted for if the samples of thiamine used were impure. However, recrystallization of the thiamine from methanol–ethanol failed to alter the amount of CO_2 evolved. Further, when pyridine was used as acceptor, about 0·95 mole of the expected product, heteropyrithiamine, was formed per mole of thiamine destroyed, suggesting that the thiamine was at least 95% pure. A possible reason for the low outputs is that a small amount of thiamine is destroyed under the manometric conditions by a mechanism that does not lead to the release of H^+ ion. This is supported by the results obtained when thiamine was present in excess and piperidine was limiting, for somewhat higher results were then obtained.

Proof that the transfer reaction is the major one under the manometric conditions was given by the isolation, in reasonably good yield, of the pyrimidinylmethylpiperidine product from a large-scale reaction mixture.

To 150 mg. of thiamine chloride hydrochloride, 13·5 ml. of 0·2 M-$NaHCO_3$ and 4·5 ml. of M-piperidine hydrochloride (pH 7·5) in a total volume of 86 ml., enzyme *A* (4 ml.) was added. The mixture was gassed with $N_2 + CO_2$ (95:5, v/v), a few drops of $CHCl_3$ were added towards the end of the gassing, and the vessel was sealed. After the mixture had been kept at 37° for 8 hr., 10 ml. of 50% (w/v) metaphosphoric acid was added and the precipitate removed by centrifuging. The supernatant was made strongly alkaline with 10 N-NaOH and extracted continuously with ether for 24 hr. The ether extract was evaporated to dryness *in vacuo*. The pale-yellow residue was recrystallized from aqueous ethanol, to give colourless crystals which dried *in vacuo* over conc. H_2SO_4, forming a white solid. The m.p. was 96°. The mixed m.p. with an authentic sample of *N*-(4-amino-2-methylpyrimidin-5-yl)methylpiperidine (m.p. 97°) was 97°. The weight was 43·3 mg., representing a 53·5% yield, based on the thiamine added.

Effect of varying different factors on the activity of bracken thiaminase

Different amines. The activity of a number of different amines in the transfer reaction catalysed by bracken thiaminase has been tested by the manometric method and the results are shown in Table 3. A number of the amines tested are known plant constituents, and with the exception of ammonia and *m*-aminobenzoic acid, which gave inconclusive results, they were all active in the transfer reaction. The extent of CO_2 binding which might be brought about by the presence of amines and hence lead to a low output was not determined

Table 3. *Effect of different amines on the rate of the thiaminase reaction*

The standard conditions were used (see Materials and Methods). The thiamine concentration was 0·005 M and the amines, previously adjusted to pH 7·5 with HCl, were present at a final concentration of 0·05 M. Thiaminase preparation *A* (0·3 ml.) was used as enzyme. The values given are corrected for the blank outputs obtained when thiamine was omitted or when enzyme previously heated at 100° for 15 min. was used. The blank values did not exceed 8 μl. in 1 hr. The values are not corrected for CO_2 retention.

Amine	Output of CO_2 in 1 hr. (μl.)
Trimethylamine	280
Triethylamine	12·5
Piperidine	160
N-Methylpiperidine	13
Diethylamine	100
Aniline	152
Tyramine	130
Ethanolamine	121
Taurine	98
Tryptamine	78
m-Aminobenzoic acid	3
Putrescine	26
Ammonia	0

Table 4. *Effect of varying the concentration of piperidine and trimethylamine on the rate of the thiaminase reaction*

The standard conditions were used (see Materials and Methods). The thiamine concentration was 0·005 M and the amine concentration was varied. Thiaminase preparation *A* was used as enzyme, 0·3 ml. with piperidine and 0·1 ml. with trimethylamine. The reaction velocity was calculated from the output in the first 30 min. of the reaction. Blank uptakes when thiamine was omitted or when heated enzyme (15 min. at 100°) was used were less than 3 μl. of CO_2 in 30 min. Values are corrected for CO_2 retention.

Concn. of amine (M)	Output of CO_2 (μl./hr.)	
	Piperidine	Trimethylamine
0	0	0
0·0025	68·5	3·5
0·01	127	48
0·025	156	—
0·05	163	187·5
0·075	156	—
0·2	132	—
0·3	—	570
0·5	103	—

experimentally with all of the amines. With aniline, piperidine and trimethylamine the values for retention of CO_2 did not exceed 11% and it is unlikely that values much greater than this would have been obtained for the other amines.

Amine concentration. The effect of varying the concentration of piperidine and trimethylamine on the velocity of the reaction is shown in Table 4. With piperidine, the results show that the velocity increases with increase in piperidine concentration up to about 0·025 M but decreases with relatively high piperidine concentrations. The results also show that the velocity of the reaction falls off with decrease in trimethylamine concentration much more sharply than with decrease in piperidine concentration.

Table 5. *Effect of varying the thiamine concentration on the rate of the thiaminase reaction*

The standard conditions were used. The piperidine concentration was 0·05 M and the thiamine concentration was varied. Thiaminase preparation *A* (0·2 ml.) was used as enzyme. The reaction velocity was calculated from the output in the first 30 min. of the reaction. Blank uptakes when thiamine or piperidine were omitted were less than 2·5 μl. in 30 min. The values are not corrected for CO_2 retention but from the results of the other experiments this would not be greater than about 11%.

Concn. of thiamine (M)	Output of CO_2 (μl./hr.)
0·001	68·5
0·002	86
0·004	105·5
0·006	107
0·008	116
0·016	123
0·024	129

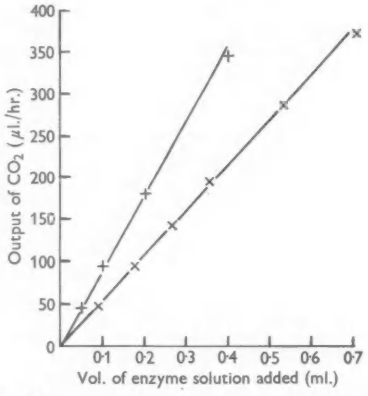

Fig. 2. Effect of varying the amount of enzyme on the rate of evolution of CO_2. The standard conditions were used with 0·005 M-thiamine. ×, Piperidine (0·05 M) and enzyme *A*; +, trimethylamine (0·05 M) and enzyme *A* diluted with 1 vol. of water. The rate of CO_2 evolution was constant over the first 20–25 min. of the reaction and it is from measurements in this period that the rates plotted were calculated. The values are corrected for the blank uptakes (1–6 μl./hr.) when thiamine was omitted. The values are not corrected for CO_2 retention, but experience with similar mixtures suggests that such retention would not be greater than 10%.

Thiamine concentration. The effect of varying the thiamine concentration with piperidine as acceptor is shown in Table 5. The results show that comparatively large increases in reaction velocity follow an increase in thiamine concentration up to about 0·005 M but that further increase in thiamine concentration brings about only a small increase in the velocity.

Effect of varying the amount of enzyme. Under the standard conditions, with either piperidine or trimethylamine as acceptor, the rate of evolution of CO_2 is directly proportional to the amount of enzyme used, provided that the velocity does not exceed about 350 μl. of CO_2/hr. (Fig. 2). The rates of evolution of CO_2 with piperidine and trimethylamine are 24 and 78 μmoles/ml. of enzyme *A*/hr. respectively. With pyridine as acceptor, under the conditions used by Kenten (1957) for the estimation of thiaminase activity, the rate of formation of heteropyrithiamine was 650 μmoles/ml. of enzyme *A*/hr. The activity of enzyme *A* with pyridine as acceptor is therefore some 8 times that with trimethylamine and about 27 times that with piperidine. By using some of the thiaminase preparations of Kenten (1957) it was found that, on average, the rate with pyridine was 26 times that with piperidine manometrically.

Activity of bracken thiaminase towards thiamine analogues. The activity of bracken thiaminase towards a number of compounds having structural similarities to thiamine has been tested manometrically and the results are given in Table 6. Piperidine was used throughout as the acceptor amine. In calculating the expected output when compounds other than thiamine are tested as substrates, the same considerations apply as those discussed previously, namely that it is the extent of ionization of the acceptor amine and the products, as well as their alteration in concentration, which will control the CO_2 output. Heteropyrithiamine and quinilinothiamine differ from thiamine in that the thiazole residue is replaced by pyridine and quinoline respectively. Hence with piperidine (pK_a 10·8 at 37°) as acceptor the transfer reaction would give the same pyrimidine–methylpiperidine compound as that obtained with thiamine, but, instead of a thiazole compound, pyridine or quinoline would be released. Since neither pyridine nor quinoline would be significantly ionized at pH 7·5,

the thiaminase reaction should lead to an output of 1·25 moles of CO_2/mole of heteropyrithiamine or quinilinothiamine destroyed (see Table 1). With oxythiamine, which differs from thiamine in that the primary amino group attached to the pyrimidine residue is replaced by hydroxyl, similar considerations apply. With the benzyl compounds, the pK_a of the benzylpiperidine compounds which would be formed if thiaminase action took place is not known; if they were very weak bases an output of 2 moles of CO_2 would be expected [equation (5)], and if strong bases, 1 mole of CO_2/mole of benzylthiazolium compound destroyed [equation (4)]. The failure to demonstrate thiaminase activity towards the benzylthiazolium compounds is therefore almost certainly because they are not attacked by the enzyme under the conditions used. It is also clear that both quinilinothiamine and pyrithiamine are readily attacked by the enzyme in the presence but not in the absence of piperidine, suggesting that, as with thiamine (Kenten, 1957), the enzyme does not easily catalyse the hydrolytic fission of these compounds. With oxythiamine a small but definite activity was found. The activity was the same with two different preparations of oxythiamine and was unchanged after these had been recrystallized twice. Rydon (1951) reports that oxythiamine, made by his method, contains not more than 0·00015% of thiamine, which amount would be insufficient to give a measurable output of CO_2 under the conditions used with oxythiamine.

At a concentration of 0·05 M-piperidine the maximum rate of thiaminase action is nearly reached at 0·005 M-thiamine (Table 5). Under these conditions the addition of either 0·005 M-heteropyrithiamine or 0·005 M-quinolinothiamine failed to bring about an appreciable increase in the rate of evolution of CO_2, suggesting that thiaminase is involved in the attack on all three compounds. Similarly the presence of oxythiamine in addition to thiamine did not affect the rate of evolution of CO_2; this suggests that oxythiamine has a low affinity for the enzyme, but because of the low activity with oxythiamine alone this cannot be used as evidence that thiaminase attacks oxythiamine. The presence of the *o*-nitrobenzylthiazolium compounds was without appreciable effect on the activity of thiaminase towards thiamine, but the *o*-aminobenzylthiazolium compound (ABMT) inhibited strongly. Under the conditions used in Table 6 the inhibition by this compound increased with time until, after about 30 min., it was virtually complete.

The effect of ABMT on the activity of bracken thiaminase is further illustrated in Fig. 3. The results show that if the enzyme preparation is incubated with the inhibitor before the thiamine is added the amount of inhibition obtained in the first 20–30 min. of the reaction is increased. How-

Table 6. *Activity of bracken thiaminase towards thiamine analogues*

The standard conditions and enzyme *B* (0·55 ml.) or *C* (0·4 ml.) were used. The piperidine concn. was 0·05 M and thiamine and thiamine analogue concn. 0·005 M. When the effect of the thiamine analogues on the rate of reaction with thiamine was tested, with the exception of compound E, which was used at 0·0005 M, both compounds were present at 0·005 M final concn. The values are corrected for the blank uptakes when thiamine or analogue was omitted (4–7 μl.). Heating the enzyme for 15 min. at 100° abolished over 95% of the activity towards compounds A, B, C and D. The values are not corrected for CO_2 retention; this was determined with some of the systems and did not exceed 10% of the experimental output.

	Compound	Output of CO_2: With piperidine (μl. of CO_2/hr.)*	With piperidine (μl. of CO_2 in 3 hr.)	Without piperidine (μl. of CO_2 in 3 hr.)
A	Thiamine	149	293	5
B	Heteropyrithiamine	86	191	3
C	Quinilinothiamine	80	198	0
D	Oxythiamine	10	27	3·5
E	3-(*o*-Aminobenzyl)-4-methylthiazolium chloride	—	3	—
F	4-Methyl-3-(*o*-nitrobenzyl)thiazolium chloride	—	−1·5	—
G	5-(β-Hydroxyethyl)-4-methyl-3-(*o*-nitrobenzyl)thiazolium chloride	—	2·5	—
A+B		158	—	—
A+C		149	—	—
A+D		141	—	—
A+E (0·0005 M)		30†	—	—
A+F		159	—	—
A+G		138·5	—	—

* Calculated from measurements in the first 30 min. of the reaction. † Evolution of CO_2 in first hr.

ever, this difference diminishes with time and after 20–30 min. the rates of reaction are about the same whether the enzyme has, or has not, been previously incubated with the inhibitor. ABMT is a powerful inhibitor of the bracken-thiaminase reaction. Under the conditions of Fig. 3, when the enzyme preparation was incubated with the inhibitor at 2 μM before thiamine was added the reaction velocity was diminished by 15–20%; at a concentration of 50 μM the inhibition was almost complete. Increasing the concentration of thiamine was without appreciable effect on the amount of inhibition (Table 7).

DISCUSSION

The present work shows that the manometric method is suitable for studying the action of bracken thiaminase. The calculated data of Table 1 show that for most systems an evolution of CO_2 would be expected as a result of thiaminase action. The quantitative studies with aniline, pyridine, piperidine and trimethylamine suggest that, although (under the conditions used when thiamine was limiting) some 5–10% of the thiamine may be lost in side reactions, there is a reasonable agreement between the calculated and experimental outputs of CO_2. Sealock & Livermore (1944) demonstrated that an output of CO_2 followed the action of a preparation of thiaminase from carp viscera on thiamine in bicarbonate solution. They obtained outputs varying from 0·57 to 0·86 mole of CO_2/mole of thiamine destroyed. With the exception of the thiaminase produced by a bacterium (Fujita, Nose & Kuratani, 1954) which catalyses the hydrolytic fission of the methylene–quaternary nitrogen bond of thiamine, most of the thiaminases have been shown to catalyse the transfer reaction of equation (1). It appears probable therefore that the manometric method may prove useful in studies of other thiaminases.

In the present work it has been shown that a number of amines, many of which are known plant constituents, are active in the bracken-thiaminase-catalysed transfer reaction. Although Fujita *et al.* (1952*a*) found that taurine failed to increase the rate of thiamine destruction by crude preparations of thiaminase from shellfish, fish and bacteria, the present work shows (Table 3) that this amino compound is reasonably active with bracken thiaminase. When thiamine is added to an aqueous suspension of ground Quahog clams, thiaminase action leads to the formation of icthiamine

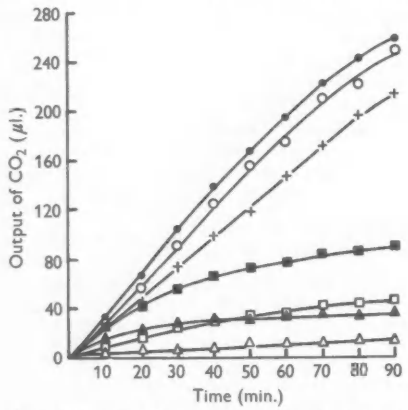

Fig. 3. Effect of 3-(*o*-aminobenzyl)-4-methylthiazolium chloride (ABMT) on the activity of bracken thiaminase. The experiments were made under the standard conditions with a thiamine concn. of 0·005 M. In one experiment the enzyme *A* (0·05 ml.) was incubated with ABMT in the presence of 0·1 M-trimethylamine and 0·05 M-$NaHCO_3$ in the main vessel for 20 min. at approx. 20° and then for 15 min. at 37° before the thiamine (0·005 M final concn.) was added from the side arm. In the other experiment thiamine (0·005 M), trimethylamine (0·1 M), $NaHCO_3$ (0·05 M) and ABMT were in the main vessel and enzyme *A* (0·05 ml.) was added from the side arm after 15 min. at 37°. Enzyme added from the side arm; ●, no ABMT; ■, 10 μM-ABMT; ▲, 50 μM-ABMT. Enzyme incubated with ABMT, thiamine added from side arm; ○, no ABMT; +, 2 μM-ABMT; □, 10 μM-ABMT; △, 50 μM-ABMT. The values given are corrected for blank uptakes in the absence of thiamine; these were less than 7·5 μl. in 90 min. The values are corrected for CO_2 retention, which was found to be 7% with the system containing the largest amount of ABMT.

Table 7. *Effect of varying the thiamine concentration on the inhibition of bracken thiaminase by* 3-(*o-aminobenzyl*)-4-*methylthiazolium chloride* (*ABMT*)

The conditions used were those of Fig. 3, the enzyme being incubated with 4 μM-ABMT in the main vessel along with the other reactants for 30 min. at room temp. and then for 15 min. at 37° before adding thiamine from the side arm. Under these conditions the rate of evolution of CO_2 was almost constant over the first 20 min. of the reaction and the values given below were calculated from measurements in this period. The values are not corrected for CO_2 retention. With boiled enzyme and mixtures corresponding to the highest thiamine concentration, CO_2 retention was estimated to be 7%. The values given are corrected for the rates in the absence of thiamine, these were less than 6 μl./hr.

Concn. of thiamine (mM)	Output of CO_2 (μl./hr.) Without inhibitor	With inhibitor	Inhibition (%)
0·99	132	72	45·5
1·48	166·5	78	53
2·46	183	94	49·5
6·17	214·5	98	54

(Barnhurst & Hennessy, 1952). It is likely that icthiamine is a pyrimidine–taurine compound produced by a thiaminase-transfer reaction involving taurine as the natural acceptor amine (Woolley, 1953).

Kenten (1957) proposed, by analogy with the mechanism which is thought to hold for the other transferring enzymes, that the bracken thiaminase reaction could be formulated:

$$P\text{–}CH_2\text{–}T^+ + \text{enzyme} \rightarrow P\text{–}CH_2\text{–enzyme}^+ + T$$

$$P\text{-}CH_2\text{–enzyme}^+ + R\text{-}NH_2 \rightarrow P\text{–}CH_2\text{–}NHR + H^+ + \text{enzyme}.$$

If this mechanism is correct it might be expected that amines which were not ionized would react more readily with the positively charged pyrimidine–enzyme complex. It is surprising therefore that, for example, piperidine (pK_a 10·8), which would be fully ionized under the experimental conditions, was as active as aniline (pK_a 4·4).

In agreement with the results of Kenten (1957) no evidence was obtained that the bracken-thiaminase preparations used were capable of catalysing the hydrolytic fission of thiamine. Under the conditions used such activity would have led to the evolution of CO_2:

$$P\text{–}CH_2\text{–}T^+ + H_2O \rightarrow P\text{–}CH_2\text{·}OH + T + H^+.$$

In the absence of an acceptor amine the CO_2 outputs obtained were extremely small and it must be concluded that if hydrolytic activity were present it must be neglible in comparison with the transfer activity of the preparations.

It is shown that with both trimethylamine and piperidine as acceptor amines, the rate of evolution of CO_2 is directly proportional to the amount of bracken thiaminase preparation added. Although with these amines under the manometric conditions the thiaminase activity is less than when pyridine is used as acceptor under the conditions of Kenten (1957), it appears that the manometric method should be of use for the estimation of thiaminase activity.

Inhibition of thiaminase by ABMT would be expected to be competitive because of its structural resemblance to thiamine. With fish thiaminase Sealock & Goodland (1944) have shown that ABMT is a competitive inhibitor. The extent of inhibition, however, was found to vary with the particular thiaminase preparation used and a second factor, possibly Mn^{2+} ion, was implicated in the binding of the inhibitor to the enzyme (Sealock & Livermore, 1949). With bracken thiaminase, ABMT inhibits very strongly but the inhibition is not competitive, for, increasing the concentration of thiamine fails to decrease the amount of inhibition. Also, the amount of inhibition increases with increase in time of exposure of the thiaminase to ABMT. It is possible that the ABMT initially combines reversibly with the enzyme at the same site as thiamine but that a second irreversible reaction follows which gives an inactive ABMT–enzyme complex. Another possibility is that under the experimental conditions ABMT is altered in some way to give a powerful, but non-competitive, inhibitor of bracken thiaminase.

Fujita *et al.* (1955) have shown that heteropyrithiamine and quinilinothiamine are substrates of bracken thiaminase. They demonstrated the formation of thiamine when these compounds were incubated with the thiazole component of thiamine as acceptor amine in the presence of bracken thiaminase. The reaction did not proceed readily under the conditions used by Fujita *et al.*, and after 40 hr. at 50° only 1–3 % of the expected amount of thiamine was formed. In the present work it is shown that both heteropyrithiamine and quinilinothiamine are readily attacked by bracken thiaminase and that this enzyme may also have a slight activity towards oxythiamine.

These results show that the manometric method can be used to test the activity of thiaminase towards structural analogues of thiamine. The method is therefore likely to be of value in testing the action of thiamine on compounds for which analytical methods are not available.

An advantage of the manometric method is that the variation of the rate of the reaction with time can be kept under continuous observation. With other methods this would involve the analysis of a great many samples. The method does suffer from the disadvantage that only a small range of pH can be usefully studied with bicarbonate–carbon dioxide buffer, and a further disadvantage is that the ionization constants of the reactants and products must be known in order to calculate the expected evolution of CO_2.

The bearing of the present results on thiaminase action *in vivo* is not clear since its function is not understood. The possible physiological functions of thiaminase have been discussed by Woolley (1953) and Kenten (1957).

SUMMARY

1. The transfer reaction of bracken thiaminase can lead to the production of H^+ ions. Under these conditions it is shown that the reaction can be studied manometrically in bicarbonate–carbon dioxide buffer.

2. When aniline, piperidine, pyridine and trimethylamine are used as acceptor amines with thiamine in limiting amount, about 5–10 % of the thiamine may be lost in side reactions, but there is a reasonable agreement between the calculated and experimental carbon dioxide output.

3. The effect on the rate of bracken thiaminase action of varying the concentration of thiamine

and the concentration and type of acceptor amine has been studied.

4. The manometric method has been used to demonstrate that heteropyrithiamine, quinilinothiamine and possibly oxythiamine are thiaminase substrates.

5. The effect of several structural analogues of thiamine on bracken thiaminase has been studied and it is shown that 3-(*o*-aminobenzyl)-4-methylthiazolium chloride is a powerful but non-competitive inhibitor.

6. Conditions are described under which the rate of evolution of carbon dioxide is directly proportional to the amount of bracken-thiaminase preparation present. It is suggested that the manometric method could be used for estimating thiaminase activity.

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Uridine Diphosphoglucose Pyrophosphorylase of Pea Seeds

By DONELLA H. TURNER and J. F. TURNER

Plant Physiology Unit, Division of Food Preservation and Transport, C.S.I.R.O., Botany School, University of Sydney, Australia

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The reaction (1) catalysed by uridine diphosphoglucose (UDPG) pyrophosphorylase involves the pyrophosphorolytic cleavage of UDPG with the formation of uridine triphosphate (UTP) and glucose 1-phosphate (G 1-P):

$$\text{UDPG} + \text{inorganic pyrophosphate} \rightleftharpoons \text{UTP} + \text{G 1-P}. \quad (1)$$

The pyrophosphorolysis of UDPG was first reported by Munch-Petersen, Kalckar, Cutolo & Smith (1953) in *Zwischenferment* preparations. UDPG pyrophosphorylase has also been found in extracts from liver (Mills, Ondarza & Smith, 1954) and mammary gland (Smith & Mills, 1955). A more detailed investigation of the properties of the enzyme was carried out by Munch-Petersen (1955) with a preparation from yeast. Burma & Mortimer (1956) obtained evidence that UDPG pyrophosphorylase was present in homogenates of sugar-beet leaves and Neufeld, Ginsburg, Putman, Fanshier & Hassid (1957) have shown that the enzyme is present in extracts from mung bean and several other plant tissues.

Experiments on the synthesis of sucrose from G 1-P and fructose had suggested that UDPG pyrophosphorylase was active in pea extracts (Turner, 1953, 1954, 1957). In the present investigation UDPG pyrophosphorylase was found in pea-seed extracts and a preparation free from interfering enzymes was obtained. Some properties of the enzyme were studied and its presence in certain other plant tissue extracts was established. The equilibrium of the reaction was examined.

MATERIALS AND METHODS

Substrates. UTP was obtained from Pabst Laboratories, Milwaukee, Wis., U.S.A.

UDPG was obtained from Pabst Laboratories and from Sigma Chemical Co., St Louis, Mo., U.S.A.

G 1-P was prepared by an unpublished method of Professor C. S. Hanes and Dr R. Hill.

Sodium pyrophosphate was analytical-reagent grade (British Drug Houses Ltd.).

Preparation of uridine diphosphoglucose pyrophosphorylase from pea seeds. A crude extract was prepared from dried pea seeds (*Pisum sativum* L.) as described by Turner (1957), and centrifuged at 20 000 *g* for 20 min. at room

temperature. The supernatant was treated with neutralized saturated $(NH_4)_2SO_4$ at 4° and the fraction obtained between 60 and 70% saturation dissolved in 7 ml. of water and dialysed with rocking at 4° for 2·5 hr. against 25 mM-phosphate buffer (KH_2PO_4–NaOH), pH 7.

The clear, colourless enzyme preparation could be stored at −15° for 3 months with only slight loss of activity. The preparation was diluted 100-fold with water before use. Heating at 60° for 10 min. resulted in 75% loss of UDPG pyrophosphorylase activity. No interfering enzymic reactions involving any of the components of the UDPG pyrophosphorylase reaction were detected. In the presence of Mg^{2+} ions, UDPG underwent a non-enzymic decomposition leading to the formation of a substance which travelled the same distance as uridine monophosphate when applied to filter paper and developed with ethanol–ammonium acetate (M, pH 7·5; 7·5:3, v/v). This decomposition was suppressed in the presence of sodium pyrophosphate.

Preparation of enzyme extracts from other plant tissues. Seeds of maize, wheat, broad bean and pea were germinated and allowed to grow for 7–10 days in damp sand under light. The roots or shoots were removed, ground with 40 mM-$NaHCO_3$ and centrifuged. The supernatant was treated with $(NH_4)_2SO_4$ to 80% saturation and the precipitate taken up in water and dialysed as described previously. Sugar-cane-stem cuttings were kept in the laboratory in damp sand for 10 days. The shoots and roots were then ground and treated in a similar manner.

Soaked wheat seeds and the laminae of leaves of silver beet (*Beta vulgaris* L. var. *cicla* Moq.) were blended with 40 mM-$NaHCO_3$. Potato tubers were sliced, minced and the juice was extracted by squeezing through muslin. The extracts of these tissues were centrifuged and treated with saturated $(NH_4)_2SO_4$. The fraction collected between 50 and 80% saturation was suspended in water and dialysed.

Preparation of inorganic pyrophosphatase. Inorganic pyrophosphatase was prepared from baker's yeast by the method of Heppel & Hilmoe (1951).

Enzyme digests. The enzyme digests were maintained at 30°. For the assay of UDPG pyrophosphorylase activity the following components were mixed: 0·5 μmole of UDPG, 2·5 μmoles of sodium pyrophosphate, 1·25 μmoles of $MgCl_2$, 50 μmoles of 2-amino-2-hydroxymethylpropane-1:3-diol (tris)–HCl buffer, pH 7·9, 0·05 ml. of enzyme; total volume, 0·25 ml. The time of incubation was 15 min. The enzyme concentration was adjusted so that not more than 0·28 μmole of UTP (corresponding to 56% conversion of UDPG) was formed in 15 min. Under these conditions the reaction rate was approximately constant during the incubation period. To show the reverse reaction (the formation of UDPG from UTP) digests of the following composition were used: 0·5 μmole of UTP, 7·5 μmoles of G 1-P, 1·25 μmoles of $MgCl_2$, 50 μmoles of tris–HCl buffer (pH 7·9), 0·05 ml. of inorganic pyrophosphatase, 0·05 ml. of enzyme; total volume, 0·25 ml.

In determining the equilibrium for the forward reaction enzymic digests of the following composition were used: 0·5 μmole of UDPG, 2·3 μmoles of sodium pyrophosphate, 6·7 μmoles of G 1-P, 1·25 μmoles of $MgCl_2$, 50 μmoles of tris–HCl (or tris–acetate) buffer of the required pH, 0·05 ml. of enzyme; total volume, 0·25 ml. G 1-P was added so that the amount of UDPG remaining at equilibrium would be convenient for estimation. The equilibrium with the reverse reaction was determined with enzymic digests of the following composition: 0·5 μmole of UTP, 6·7 μmoles of G 1-P, 2·3 μmoles of sodium pyrophosphate, 1·25 μmoles of $MgCl_2$, 50 μmoles of tris–HCl buffer (pH 7·9), 0·05 ml. of enzyme; total volume 0·25 ml. Sodium pyrophosphate was added to prevent the non-enzymic decomposition of UDPG. For determination of the equilibrium, samples were taken from the enzymic digests at 120 and 150 min.

Digest samples were inactivated by the addition of 2 vol. of ethanol or 0·25 vol. of 5% (w/w) $HClO_4$ (followed by neutralization with dil. aq. NH_3 soln.) and centrifuged at 1000 *g* for 5 min.

Detection and estimation of uridine nucleotides. The uridine nucleotides were separated on paper chromatograms, eluted with water and estimated by their absorption at 262 mμ. Descending chromatography in all-glass apparatus maintained at 25° was employed (Hanes & Isherwood, 1949). Whatman no. 3MM papers, which had been washed as chromatograms with 2N-acetic acid and water, were used.

Extracts of the enzyme digests were applied directly to the papers. A current of air was passed over the papers and up to seven applications of 24 μl. portions were made on each spot. The papers were developed for 16 hr. with the ethanol–ammonium acetate (M, pH 7·5; 7·5:3, v/v) solvent of Paladini & Leloir (1952). The nucleotides were detected by ultraviolet light with the procedure of Markham (1955) as modified by Rowan (1957), and eluted from the spot area with 3 ml. of water. The absorption was measured in a Beckman Model DU spectrophotometer and a molar absorbency index of 10×10^3 at 262 mμ used for the uridine compounds (Ploeser & Loring, 1949). Appropriate areas of paper were taken for blank values. Individual estimations of nucleotides applied to papers, developed and extracted gave recoveries within 4–5% of the theoretical.

Determination of glucose. Glucose was estimated by the method of Turner, Turner & Lee (1957).

RESULTS

Uridine diphosphoglucose pyrophosphorylase activity

The pyrophosphorolysis of UDPG by the pea-seed enzyme is shown in Fig. 1. The reaction could be followed by estimating either the formation of UTP or the disappearance of UDPG; this was in agreement with preliminary experiments which showed that there was no significant change in any of the reactants (equation 1) when these were incubated individually with the enzyme preparation.

The reverse reaction (i.e. the formation of UDPG from UTP) was demonstrated by using an enzymic digest containing UTP, G 1-P and inorganic pyrophosphatase as described above. UDPG was isolated from a digest containing an excess of UTP over G 1-P by applying ethanolic extracts as 'streaks' to Whatman no. 3MM papers which were developed in the ethanol–ammonium acetate solvent. The UDPG was eluted, hydrolysed in HCl (pH 2) for 10 min. at 100° and the solution neutralized. The ratio of free glucose to uridine was found to be 1.

Equilibrium of the reaction. The equilibrium of the UDPG pyrophosphorylase reaction was in favour of UTP formation. In determining the apparent equilibrium constant (K^1) both UTP and UDPG were estimated and the corresponding changes in G 1-P and sodium pyrophosphate (PP) calculated. The value of K^1, which is defined as

$$K^1 = \frac{[\text{UTP}].[\text{G 1-P}]}{[\text{UDPG}].[\text{PP}]}$$

was found to be 7·2 (mean of ten determinations) with enzymic digests of the composition described for the determination of the equilibrium with the forward reaction. There was no significant difference in the value of K^1 determined with the reverse reaction. Identical values were obtained after incubation for 120 and 150 min.

Variation in Mg^{2+} ion concentration affected the value of K^1: with Mg^{2+} ion concentrations of 2·5, 5 and 10 mM the equilibrium constants were 8·4, 7·2 and 3·8 respectively at pH 7·9. Change in pH of the reaction mixture also affected the value of K^1. In the presence of 2·5 mM-$MgCl_2$, K^1 was 3·5 and 8·4 at pH 7·0 and 7·9 respectively.

Effect of Mg^{2+} ions. The effect of Mg^{2+} ion concentration on the pyrophosphorolysis of UDPG is shown in Fig. 2. The optimum concentration of Mg^{2+} ions under the conditions of the experiment was approximately 5 mM. Increase of Mg^{2+} ions beyond this concentration inhibited the reaction.

Mg^{2+} ions could be replaced by Mn^{2+} ions and an identical rate of UDPG pyrophosphorolysis was obtained. The optimum concentration of Mn^{2+} ions was approximately mM. Co^{2+} and Ni^{2+} ions at concentrations of 2·5 mM also stimulated UDPG pyrophosphorylase.

Effect of concentration of substrates. The effect of increasing sodium pyrophosphate concentration on the rate of reaction is shown in Fig. 3. The optimum concentration was approximately 10 mM and further increase depressed the rate of UDPG pyrophosphorolysis. At concentrations of sodium pyrophosphate less than 5 mM some non-enzymic breakdown of UDPG occurred. Increasing the concentration of UDPG from 2 to 4 mM did not

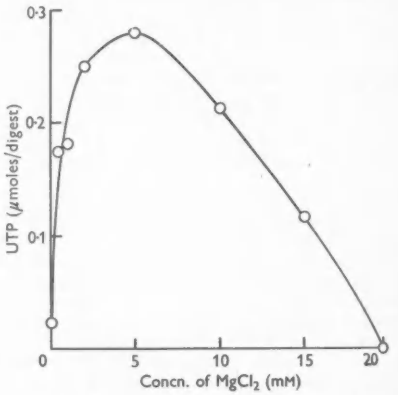

Fig. 2. Effect of $MgCl_2$ concentration on the UDPG pyrophosphorylase reaction. The enzymic digests were of the composition used for the assay of UDPG pyrophosphorylase. Concentration of $MgCl_2$ varied as shown.

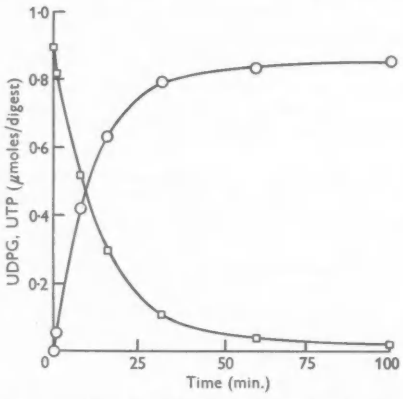

Fig. 1. Pyrophosphorolysis of UDPG and the formation of UTP. □, UDPG; ○, UTP. The enzymic digest contained 0·9 μmole of UDPG, 5 μmoles of sodium pyrophosphate, 2·5 μmoles of $MgCl_2$, 100 μmoles of tris–HCl buffer, pH 7·9, 0·1 ml. of enzyme, total volume 0·5 ml.

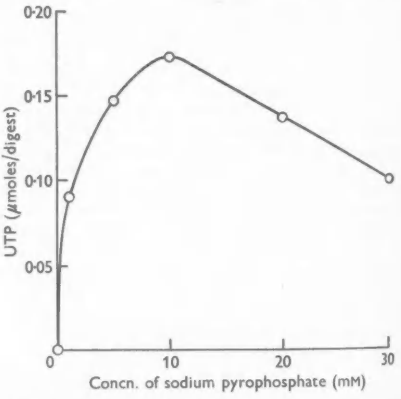

Fig. 3. Effect of concentration of sodium pyrophosphate on the UDPG pyrophosphorylase reaction. Enzymic digests of the composition described for the assay of UDPG pyrophosphorylase were used. Concentration of sodium pyrophosphate varied as shown.

affect the rate of reaction; in these experiments the initial concentration of sodium pyrophosphate was 10 mM.

Effect of pH. There was little variation in enzyme activity over the range pH 7·5–9·0. In Fig. 4 the results obtained with a series of buffers containing tris (final concentration 0·2 M) and acetic acid are shown. In separate experiments with glycine (final concentration 0·2 M)–NaOH buffers it was observed that the activity decreased by 30 % when the pH was raised from 9 to 10.

Effect of inhibitors. The following did not significantly affect the activity: fluoride, inorganic orthophosphate, fluoride plus inorganic orthophosphate, iodoacetate, or arsenate at 10 mM-concentration; *p*-chloromercuribenzoate at mM-concentration, or Hg^{2+} ions at 0·1 mM-concentration. Ethylenediaminetetra-acetate (10 mM) inhibited the reaction by 90 %.

Distribution of uridine diphosphoglucose pyrophosphorylase

Active preparations of the enzyme were obtained from all plant tissues examined. These were wheat shoots, maize shoots, broad-bean shoots, sugar-cane shoots, pea roots, sugar-cane roots, wheat seeds, silver-beet leaves and potato tubers. A phosphatase which acted on UTP to give uridine diphosphate and uridine monophosphate was present in crude extracts of pea seeds and in the extracts prepared from potato tubers, silver-beet leaves, wheat seeds and shoots, broad-bean shoots and maize shoots.

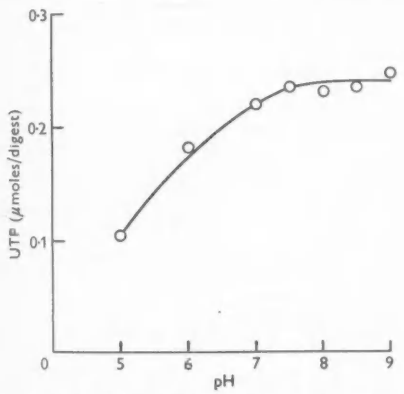

Fig. 4. Effect of pH on the UDPG pyrophosphorylase reaction. Composition of the enzymic digests was that described for the assay of UDPG pyrophosphorylase. The reaction was studied in tris (0·2 M)–acetate buffers of pH indicated.

DISCUSSION

The present investigation shows that active preparations of UDPG pyrophosphorylase free from interfering enzyme reactions may be readily obtained from pea seeds.

Under all the experimental conditions used, the equilibrium of the reaction favoured the breakdown rather than the synthesis of UDPG. With the optimum concentration (5 mM) of Mg^{2+} ions and at pH 7·9 and 30°, K^1 was 7·2. Increase in Mg^{2+} ion concentration and decrease in pH of the reaction mixture each depressed the value of K^1. Effects of Mg^{2+} ion concentration and pH on the apparent equilibrium constant have been observed with other enzymic reactions involving phosphoric compounds and Mg^{2+} ions, e.g. the adenosine triphosphate–creatine transphosphorylase reaction (Noda, Kuby & Lardy, 1954). The K^1 values reported in the present paper are not thermodynamic equilibrium constants but represent the total concentration of each component (ionic and magnesium complexes) present at equilibrium. To determine a true thermodynamic equilibrium constant it would be necessary to know the nature of the components which actually participate in the reaction. Adequate information to calculate the concentrations of the possible 'reacting' components under the experimental conditions is not available.

The values of K^1 reported here differ from the figure of approximately 1 obtained by Munch-Petersen (1955) with yeast UDPG pyrophosphorylase at pH 7·4 and unspecified temperature and Mg^{2+} ion concentration (possibly approximately 5 mM). The concentrations of UDPG and sodium pyrophosphate used differed from those in the present investigation.

From the results of the present investigation it appears likely that the thermodynamic equilibrium of the UDPG pyrophosphorylase reaction lies in the direction of UTP formation. This may support the suggestion of Kalckar (1954) that the glucose–phosphate linkage in UDPG has a higher bond energy than in free G 1-P. This hypothesis has been supported by investigations on sucrose synthesis. The equilibrium constant of the reaction

UDPG + fructose ⇌ sucrose + uridine diphosphate (2)

was found to be approximately 5 (Cardini, Leloir & Chiriboga, 1955; Leloir, 1956), whereas Doudoroff (1943) obtained an equilibrium constant of 0·05 for the reaction

G 1-P + fructose ⇌ sucrose + inorganic orthophosphate (3)

The depressing effect of high Mg^{2+} ion concentrations on the pyrophosphorolysis of UDPG may

have been due to complex formation with sodium pyrophosphate. It was observed that precipitates formed in enzymic digests where the molar concentration of Mg^{2+} ions was equal to or greater than the concentration of sodium pyrophosphate. The optimum concentration of 5 mM for Mg^{2+} ions found in the present investigation is in reasonable agreement with the value of 2 mM-Mg^{2+} ions observed by Munch-Petersen (1955) for yeast UDPG pyrophosphorylase. The ability of Mn^{2+}, Ni^{2+} and Co^{2+} ions to replace Mg^{2+} ions as cofactor for pea UDPG pyrophosphorylase suggests that the metal requirement may be for a suitable bivalent cation. This agrees with the observations of Neufeld *et al.* (1957), who found that the enzyme from mung-bean seedlings required Mg^{2+}, Mn^{2+} or Co^{2+} ions.

The enzyme was found in all plant materials examined, suggesting that its distribution may be fairly general. As UDPG is the probable precursor of sucrose in plants, the enzymic transformations of UDPG are of considerable interest. The enzyme may be of major significance in plant tissues exhibiting pronounced sugar–starch changes, where G 1-P may be incorporated into either starch or sucrose. Thus the high UDPG pyrophosphorylase activity in the pea extracts is consistent with the rapid transformations of sugar into starch observed in the developing pea seed by Turner & Turner (1957) and Turner *et al.* (1957). The present investigation supports previous suggestions (Turner, 1954, 1957) that synthesis of sucrose from G 1-P and fructose in pea extracts takes place through the intermediate formation of UDPG formed from UTP and G 1-P. Evidence that the pea extracts contained a functional glycolytic system (which could lead to the production of UTP) has been recently confirmed (Hatch & Turner, 1958).

SUMMARY

1. Uridine diphosphoglucose pyrophosphorylase, which catalyses the reaction

uridine diphosphoglucose + inorganic pyrophosphate $\rightleftharpoons$ uridine triphosphate + glucose 1-phosphate

was found in pea-seed extracts. A purified preparation free from interfering enzymes was obtained.

2. The apparent equilibrium of the reaction was in favour of uridine triphosphate formation. The apparent equilibrium constant ([uridine triphosphate] [glucose 1-phosphate]/[uridine diphosphoglucose] [inorganic pyrophosphate]) was affected by Mg^{2+} ion concentration and pH.

3. Mg^{2+} ions, which could be replaced by Mn^{2+}, Co^{2+} or Ni^{2+} ions, were essential for the reaction.

4. The effects of Mg^{2+} ion concentration, concentration of substrates, pH and inhibitors on the pyrophosphorolysis of uridine diphosphoglucose were studied.

5. Active preparations of the enzyme were obtained from a number of plant tissues.

6. The possible relationship of the reaction catalysed by the enzyme to the carbohydrate metabolism of plants has been discussed.

The work described in this paper was carried out as part of the joint research programme of the Division of Food Preservation and Transport, C.S.I.R.O., and of the Botany School, University of Sydney. The authors wish to express their indebtedness to Miss S. K. Harris and Mr J. B. Lee for technical assistance; to Dr R. N. Robertson for his interest during the course of the investigation; and to Dr J. R. Vickery, Chief, Division of Food Preservation and Transport, and Professor R. L. Crocker, Botany School, University of Sydney, in whose laboratories the work was carried out.

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Biosynthesis of Intestinal Mucins

3. FORMATION OF 'ACTIVE SULPHATE' BY CELL-FREE EXTRACTS OF SHEEP COLONIC MUCOSA*

By P. W. KENT and C. A. PASTERNAK†
Department of Biochemistry, University of Oxford

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Previous results (Pasternak, Kent & Davies, 1958) showed that $^{35}SO_4^{2-}$ ion is utilized in differing degrees by surviving intestinal tissues and that the ion is incorporated as sulphate esters of intestinal mucins (Pasternak & Kent, 1958). The incorporation is energy-dependent and inhibitors of respiration also prevent the uptake of sulphate. A relatively high level of incorporation of SO_4^{2-} ion was found in colonic mucosa, and on account of its availability and ease of homogenization this tissue was selected for an investigation of the individual stages of the sulphation mechanism.

In liver, formation of phenolic sulphates involves the enzymic activation of SO_4^{2-} ions by combination with adenosine triphosphate (ATP), forming adenosine 3′-phosphate 5′-phosphosulphate (Hilz & Lipmann, 1955). D'Abramo & Lipmann (1957) secured, from chick-embryo condyles, cell-free preparations which promote the incorporation of $^{35}SO_4^{2-}$ ion into chondroitin sulphate, with indications that a similar sulphate-activation mechanism participates.

MATERIALS AND METHODS

Preparation of tissue homogenates. A portion of the large intestine (approx. 30 cm. from the anus) of adult sheep was obtained immediately after slaughter and placed in ice-cold saline. Within 30 min. the intestine was emptied, rinsed with water and transferred to ice-cold KCl solution (0·9%) brought to pH 7·5 with a little 2-amino-2-hydroxymethylpropane-1:3-diol (tris). The mucosa (12·5 g.), removed by scraping with a scalpel, was immersed in 25 ml. of 0·25 M-sucrose or KCl (0·9%) at 0°, each solution being brought to pH 8·5 by addition of tris. The tissue was then homogenized (12 strokes) in a chilled Potter-type stainless-steel apparatus.

Fractionation of homogenates. The homogenate (37 ml.) was centrifuged at 0° for 20 min. at 24 000 *g*. The clear supernatant fraction (22 ml.) was decanted and used in the experiments described below. The precipitate was resuspended in 12 ml. of 0·25 M-sucrose before investigation.

Incubations. Experiments (Table 1) were performed under O_2 in Warburg manometer flasks at 37° for 30–60 min., the centre well containing NaOH on filter paper. Each flask contained 0·1 M-sodium phosphate buffer (pH 7·6, 0·4 ml.), 0·2 M-$MgCl_2$ (0·2 ml.), 0·154 M-$MgSO_4$ (0·036 ml.), 0·02 M-ATP (0·2 ml.), 10 μc of $Na_2{}^{35}SO_4$ (approx. 0·1 ml.) and 0·2 ml. of a solution containing 0·5 M-sodium pyruvate and 0·04 M-sodium fumarate. In each case 3 ml. of a suspension of mucosal scrapings or other enzyme fraction was added. In appropriate cases, 0·5 M-sodium fluoride (0·3 ml.) was added also. Subsequent experiments (Tables 2–4) with an appropriate buffer solution, additions and supernatant enzyme fraction, were performed in stoppered tubes. The protein content of enzyme preparations (1 ml.) was determined separately by precipitation with 1 ml. of trichloroacetic acid (10%, w/v). The filtered trichloroacetic acid insoluble precipitate was weighed after being dried at 100° for 12 hr. The contents of each flask, at the end of incubation, were heated to 100° for 1 min. or were treated with ethanol (1 ml.). After centrifuging (2000 rev./min.) for 2 min. the supernatant solution was examined by paper electrophoresis and paper chromatography. (Imidazole buffers were prepared by the method of Klemperer, 1955.)

Uridine diphosphate glucose. This was obtained from California Biochemicals Inc., Berkeley, Calif., U.S.A.

Uridine diphosphate N-acetyl-D-glucosamine. This was kindly given by Dr Pontis Videla.

Paper electrophoresis. Buffers employed were: sodium diethylbarbiturate (pH 8·8, 0·07 M), ammonium acetate (pH 5·6, 0·1 M) and sodium citrate (pH 5·6, 0·1 M). At low voltage (4·3 v/cm.), experiments were carried out on Whatman no. 1 paper held between glass plates. At the higher voltage (17 v/cm.), Whatman no. 3 paper bathed in chlorobenzene (Cremer & Tiselius, 1950) was employed and the separations were carried out at 3°. Ethanediol (30 ml.) was added to the buffer (500 ml.) to prevent freezing. In analytical experiments 0·025–0·05 ml. of the test solution was used and in preparative experiments 1 ml. was applied across the paper. At the end of each experiment the paper was dried in warm air and the radioactive components were assayed as described previously (Pasternak & Kent, 1958).

Isolation of components separated by paper electrophoresis. The region of dried paper carrying the separated radioactive component was cut out and eluted continuously with glass-distilled water until the radioactivity of the paper had fallen to background count. This was usually achieved within 4 hr. The eluate was concentrated to dryness by lyophilization. Though efficient separations of $^{35}SO_4^{2-}$ ion and other ^{35}S-labelled products was achieved by the use of

* Part 2. Pasternak & Kent (1958).

† Present address: Department of Pharmacology, School of Medicine, Yale University, New Haven, Conn., U.S.A.

any of the buffers described, ammonium acetate was employed in preparative experiments since it could be removed easily during the freeze-drying procedure, provided that a trap containing solid KOH was attached to the apparatus to maintain neutrality of the specimen.

Paper chromatography. Systems employed were: (*a*) butan-1-ol–ethanol–water (water-poor phase, 4:1:5, by vol.) in descending elution (Partridge, 1946); (*b*) ethanol–M-ammonium acetate (5:2, v/v), descending elution (Paladini & Leloir, 1952); (*c*) propan-2-ol–5·7N-HCl (13:7, v/v), descending elution (Wyatt, 1951). Whatman no. 1 paper was used throughout; reducing sugars were detected with aniline hydrogen phthalate (Partridge, 1949), u.v.-absorbing substances by contact photography (Markham & Smith, 1949) and phosphorylated substances as described by Hanes & Isherwood (1949). Sulphated phenols were located as described by Loewi & Kent (1957).

Glucose, glucose 6-sulphate and SO_4^{2-} ions were conveniently separated by ascending chromatography in ethanol–0·1M-ammonium acetate (4:1, v/v) (Cohen & Scott, 1950), in which the R_F values were 0·74, 0·65 and 0·27 respectively.

Radioactive Assays. These were as described by Pasternak *et al.* (1958).

RESULTS

A preliminary survey was made of the enzymic activity of whole mucosal scrapings, whole homogenate, and of the soluble and insoluble fractions of tissue homogenates. In each case the ^{35}S-labelled products were examined by paper electrophoresis and the q_{O_2} values were measured during incubation (Table 1) and expressed as μl. of O_2/hr./mg. of trichloroacetic acid-insoluble material. By electrophoresis at pH 8·8 in diethylbarbiturate buffer three radioactive anionic substances were detected, (1) A fast-moving component which was shown to be unchanged $^{35}SO_4^{2-}$ ions, (2) an immobile component, ^{35}S-mucin and (3) an intermediately mobile ^{35}S-labelled substance, designated component *A*. It appears from Table 1 that the supernatant enzyme fraction is the most active in fixing inorganic sulphate and that the formation of component *A* is inhibited by F^- ion. The results obtained from homogenates prepared in potassium chloride or sucrose are shown in Fig. 1. It is clear that either medium is suitable and that an incubation period of 20 min. is the optimum time for the production of the labelled component *A* under the conditions of the experiment. Subsequently all the experiments were performed with potassium chloride homogenates.

Table 1. *Incorporation of* [^{35}S]*sulphate by, and* q_{O_2} *values of, preparations of sheep colonic mucosa*

All figures are averages of duplicate experiments.

Incubation medium was that described in the Methods section (containing 85 000 counts/min.).

[^{35}S]Mucin and ^{35}S-labelled component *A* were determined after incubation for 30 min. by assay of ^{35}S after paper electrophoresis (sodium diethylbarbiturate buffer) and are expressed as counts/100 000 added/mg. of protein. q_{O_2} refers to μl. of O_2/mg. (dry wt.) of trichloroacetic acid-insoluble material/hr.

	[^{35}S]Mucin	[^{35}S]-Component *A*	q_{O_2}
With fluoride			
Scrapings	3	12	−2·5
Homogenate	10	0	−3·2
Superantant	10	198	0·0
Sediment	1	0	0·0
Without fluoride			
Scrapings	62	5	−9·8
Homogenate	10	10	−2·4
Supernatant	9	463	0·0
Sediment	3	0	0·0

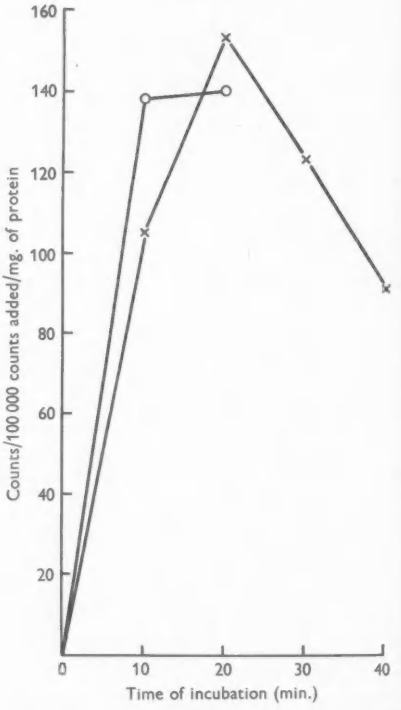

Fig. 1. Formation of ^{35}S-labelled component *A* by sheep colonic mucosa homogenized in 0·9% KCl (○) and 0·25M-sucrose (×). The incubation medium contained 10 μmoles of ATP, 4 μmoles of $MgCl_2$, 110 μmoles of $MgSO_4$, 80 μmoles of phosphate buffer (pH 7·6), 24 μc of $Na_2{}^{35}SO_4$, 7·5–12 mg. in 3 ml. of supernatant enzyme fraction in 7 ml. (total volume). ^{35}S-Labelled component *A* was determined by assay of ^{35}S after paper electrophoresis (sodium diethylbarbiturate buffer).

Table 2. *Formation of* [^{35}S]*component A by supernatant fraction of homogenized sheep colonic mucosa*

Incubation medium: 100 μmoles of tris, pH 8; 20 μc of $Na_2{}^{35}SO_4$, 10·7 mg. of enzyme in 1 ml., incubated for 20 min. at 37°. ^{35}S-labelled component *A* was determined by assays of ^{35}S after paper electrophoresis (ammonium acetate buffer) and is expressed as counts/100 000 added/mg. of trichloroacetic acid-insoluble material.

Additions	^{35}S-labelled component *A* (counts)
No addition	0
$MgCl_2$ (6·5 μmoles)	0
ATP (5·0 μmoles)	1200
$MgCl_2$ (6·5 μmoles) + ATP (5 μmoles)	2400
$MgCl_2$ (6·5 μmoles) + ATP (5 μmoles) (heated inactivated enzyme)	36

As a result of attempts to simplify the incubation medium, it was found (Table 2) that ATP and Mg^{2+} ions appear to be essential for the formation of the intermediate. In the boiled-enzyme control or ethanol-inactivated control no intermediate was formed.

In the light of these results, incubation of ATP, $^{35}SO_4{}^{2-}$ and Mg^{2+} ions with the supernatant enzyme fraction was carried out on a preparative scale and the ^{35}S-labelled component *A* was isolated. The substance had $\lambda_{max.}$ at 255 mμ (0·01 N-HCl). It proved to be markedly unstable and was readily hydrolysed by acidic conditions with the liberation of $SO_4{}^{2-}$ ions. Hydrolysis occurred even during the freeze-drying stage of its isolation unless precautions were taken to maintain neutrality during the process. During attempted preparative electrophoretic separations in sodium citrate at 17 v/cm., a second labelled intermediate (component *B*) was detected. This was less radioactive and moved more slowly than *A* but was not investigated further.

Complete release of sulphate resulted when component *A* was heated with 0·1 N-HCl at 37° for 30 min. (Fig. 2) or with 0·1 N-HCl at 100° for 15 min. but the compound appeared to be stable to 0·01 N-HCl at 100° for several minutes. Acid hydrolysis yielded, in addition to $SO_4{}^{2-}$ ions, a compound having a u.v.-absorption maximum at 260 mμ in 0·01 N-HCl.

The product of acid hydrolysis moved more slowly than component *A* in paper electrophoresis at pH 5·6 and had a mobility similar to that of adenosine diphosphate (ADP). The u.v. spectrum was unaffected by addition of bromine and the product was therefore considered not to be a uridine nucleotide (Caputto, Leloir, Cardini & Paladini, 1950). More vigorous hydrolysis of component *A* with 2·5 N-HCl at 100° for 6 hr., followed by paper-chromatographic examination of the products (freed from HCl under high vacuum) in propan-2-ol–HCl, revealed the presence of adenine (R_F 0·27, $\lambda_{max.}$ 257 mμ in 0·01 N-HCl). Under these conditions inorganic phosphate was also released.

Transfer of sulphate from component A. In order to test whether component *A* was an 'active sulphate', its reactions with phenol and *o*-nitrophenol were examined with a liver transferase system (DeMeio & Tkacz, 1950). The enzyme preparation was the supernatant fraction prepared from rat liver (4 g.), homogenized in 20 ml. of 0·9% potassium chloride and centrifuged at 2400 *g* for 30 min. at 0°; 18 ml. of supernatant was obtained, of which 0·7–1 ml. was used in each test. In the presence of this enzyme preparation ^{35}S-labelled component *A*, transfers its sulphate group to an added phenol (Table 3) in the absence of

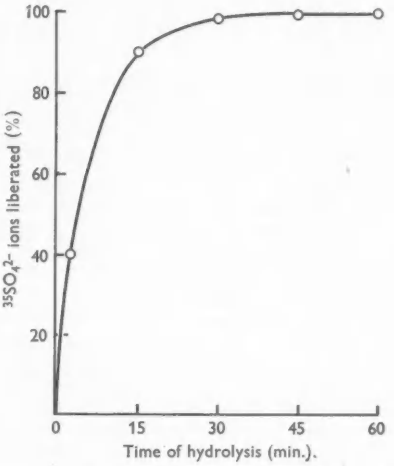

Fig. 2. Rate of hydrolysis of ^{35}S-labelled component *A* by 0·1 N-HCl at 37°. $^{35}SO_4{}^{2-}$ ions were estimated after separation by paper electrophoresis in ammonium acetate.

Table 3. *Formation of phenyl* [^{35}S]*sulphate by supernatant fraction of homogenized rat liver*

Incubation medium: 10 μmoles of $MgSO_4$, 2·5 μmoles of $MgCl_2$, 10 μmoles of cysteine, 1 μmole of phenol, 200 μmoles of tris, pH 8; 5 mg. of enzyme in 1 ml., incubated for 30 min. Phenyl [^{35}S]sulphate was determined by assay of ^{35}S after paper chromatography (butanol–ethanol–water) and is expressed as counts/100 000 added/mg. of trichloroacetic acid-insoluble material.

Additions	Phenyl [^{35}S]sulphate (counts)
$Na_2{}^{35}SO_4$ (2 μc)	0
$Na_2{}^{35}SO_4$ (2 μc) + ATP (10 μmoles)	600
^{35}S-labelled component *A* (200 μm-moles)	9400

ATP, without the intervention of inorganic sulphate. It was also found (Table 4) that the supernatant enzyme fraction from sheep colonic mucosa catalysed the formation of phenolic sulphates from component *A*.

Attempted sulphation of carbohydrate substrates. A series of experiments was performed in which isolated ^{35}S-labelled component *A* (or $^{35}SO_4^{2-}$ ions, ATP and Mg^{2+} ions) and supernatant mucosal enzyme preparation were incubated with a number of potential carbohydrate acceptors. The latter were derivatives of sugars which are either known, or related to known constituents of sulphated mucins (Werner, 1953), and included D-glucose, D-glucosamine hydrochloride, D-galactosamine hydrochloride, *N*-acetyl-D-glucosamine, methyl *N*-acetyl-α-D-galactosaminide, methyl *N*-acetyl-β-D-glucosaminide, α-D-glucose 1-phosphate, D-glucosamine 6-*O*-phosphate, uridine diphosphate glucose, uridine diphosphate *N*-acetyl-D-glucosamine and hyalobiuronic acid. The extruded and extracted guinea-pig colonic mucins (5 mg.) (Pasternak *et al.* 1958) were also examined. The following system was used to investigate the sulphation of these substances: the carbohydrates (1 μmole), cysteine (10 μmoles), imidazole (100 μmoles), $Na_2{}^{35}SO_4$ (5–20 μc) and 8–12 mg. of enzyme, in a final volume of 0·6–1·0 ml. (pH 7). After incubation for 60–90 min. at 37°, the products were examined by electrophoresis in ammonium acetate and by chromatography in ethanol–ammonium acetate. No evidence was obtained of the formation of a sulphated carbohydrate, either with the supernatant enzyme or with whole mucosal homogenates. However, added glucose 6-*O*-sulphate is still present after incubation with these enzyme preparations.

Table 4. *Formation of phenyl [^{35}S]sulphate from component A by supernatant fraction of rat liver and sheep colon*

Supernatant fractions of homogenized liver and colonic mucosa were prepared as described in the Methods section. Incubation medium 10 μmoles of $MgSO_4$, 2·5–5 μmoles of $MgCl_2$, 10 μmoles of cysteine, 1 μmole of substrate, 100–200 μmoles of imidazole or tris buffer, 0·3–20 μmoles of ^{35}S-labelled intermediate, 3·4–10·7 mg. of trichloroacetic acid-insoluble material in 0·7–1·0 ml. of enzyme preparation, incubated for 30–60 min. [^{35}S]Sulphate substrate was determined by assay of ^{35}S after paper chromatography (butanol–ethanol–water or ethanol–ammonium acetate) and is expressed as counts/100 000 added/mg. of protein.

Supernatant fraction	Substrate	pH	[^{35}S]Sulphated substrate (counts)
Rat liver	Phenol	8·0	9400
Rat liver	*o*-Nitrophenol	7·5	8600
Sheep colonic mucosa	Phenol	8·0	7600
Sheep colonic mucosa	*o*-Nitrophenol	7·0	3900

Table 5. *Spectral data of non-radioactive nucleoside components of reaction mixture*

Reaction mixture: 5 μmoles of ATP, 6·5 μmoles of $MgCl_2$, 100 μmoles of tris (pH 8), 20 μc of $Na_2{}^{35}SO_4$, 10·7 mg. of trichloroacetic acid-insoluble material (supernatant fraction of homogenized sheep colonic mucosa) in 1 ml., incubated for 20 min. at 37°. Chromatographic system was butanol–ethanol–water.

	R_F 0		R_F 0·12		R_F 0·23	
	0·01 N-HCl	0·01 N-NaOH	0·01 N-HCl	0·01 N-NaOH	0·01 N-HCl	0·01 N-NaOH
$\lambda_{max.}$ (mμ)	252	252	248	251	248	258
$\lambda_{min.}$ (mμ)	230	229	227	228	228	233
E_{250}/E_{260}	1·16	1·11	1·59	1·11	1·45	0·92
E_{280}/E_{260}	0·23	0·19	0·26	0·10	0·20	0·08

Table 6. *Spectral data of components of reaction mixture separated by chromatography in ethanol–ammonium acetate* (pH 7·5)

Reaction mixture: 5 μmoles of ATP, 6·5 μmoles of $MgCl_2$, 100 μmoles of tris (pH 8), 20 μc of $Na_2{}^{35}SO_4$, 10·7 mg. of trichloroacetic acid-insoluble material (supernatant fraction of homogenized sheep colonic mucosa) in 1 ml., incubated for 20 min. at 37°.

	R_F 0·23		R_F 0·67	
	0·01 N-HCl	0·01 N-NaOH	0·01 N-HCl	0·01 N-NaOH
$\lambda_{max.}$ (mμ)	249	251	249	252, 257
$\lambda_{min.}$ (mμ)	228	228	227	231
E_{250}/E_{260}	1·69	1·37	1·52	1·02
E_{280}/E_{260}	0·19	0·20	0·16	0·18

Breakdown of adenosine triphosphate by mucosal enzymes. In addition to the ^{35}S-labelled components *A* and *B*, a number of other u.v.-light-absorbing compounds were detected electrophoretically and chromatographically after the supernatant enzyme preparation from colon had acted on ATP + SO_4^{2-} ions. These products were not radioactive and their formation was observed in the absence of SO_4^{2-} ions.

Electrophoresis showed a stationary and a mobile u.v.-light-absorbing component; the former was phosphorus-free and appeared to be a mixture of nucleosides or purines. The mobile substance was a phosphate ester having $\lambda_{max.}$ at 248 mμ, $\lambda_{min.}$ at 222 mμ in 0·01 N-HCl and $\lambda_{max.}$ at 252 mμ, $\lambda_{min.}$ at 225 mμ in 0·01 N-NaOH, and behaved electrophoretically as an inosine monophosphate.

Paper chromatography of the incubation mixture in butanol–ethanol–water revealed a stationary component and two substances with R_F values of 0·12 and 0·23. The R_F values of these substances and their spectral characteristics agreed with those of inosine and hypoxanthine (Table 5). In ethanol–ammonium acetate, the inosine monophosphate (first recognized electrophoretically) had R_F 0·23, and is clearly distinguished from ATP and ADP. The isolated nucleotide had spectral data (Table 6) in agreement with published values.

DISCUSSION

Hitherto, the study of the mechanism of enzymic sulphation in mammalian systems has been confined largely to the formation of phenolic sulphates by liver enzymes. Hilz & Lipmann (1955) showed that 'active sulphate' is first formed from SO_4^{2-} ions and 2 moles of ATP (Wilson & Bandurski, 1956; Bandurski, Wilson & Squires, 1956), and that another enzyme is involved in this subsequent transfer of sulphate from the 'active' derivative to phenolic acceptors. The active sulphate was shown to be adenosine 3′-phosphate 5′-phosphosulphate (Robbins & Lipmann, 1956*a*, *b*). Similar sulphate activation has been described in yeast (Wilson & Bandurski, 1956) and in *Neurospora crassa* (Hilz & Lipmann, 1955). Liver is known to contain sulphated polysaccharides, notably those of the heparin group. The intestinal mucosa, on the other hand, are sites of high metabolic activity, producing extracellar, soluble, sulphated mucins. It is of interest therefore that here also evidence of sulphate activation has been found. The 'active sulphate', formed enzymically under conditions similar to those described for liver, is an adenine nucleotide carrying a labile sulphate group. The rate of hydrolysis of the latter is substantially greater than that of alkyl *O*- or *N*-sulphates (Meyer & Schwartz, 1950) and is suggestive of an anhydride linkage. The substance has the same electrophoretic mobility (0·6 that of SO_4^{2-} ions at pH 5·6) as adenosine 3′-phosphate 5′-phosphosulphate. The 'active sulphate' of the mucosa also closely resembles adenosine 3′-phosphate 5′-phosphosulphate in its ability to act as a sulphate donor in the formation of phenolic sulphates either by liver or by mucosal enzyme preparations. It is concluded that the active sulphate of colonic mucosa is either identical with or closely similar to adenosine 3′-phosphate 5′-phosphosulphate. The yield of phenyl sulphate from 'active sulphate' did not exceed 40% with unfractionated soluble mucosal enzymes, and was accompanied by some hydrolysis of the intermediate. Under the conditions necessary for the sulphation of phenols, no evidence of sulphation of simple mono- and di-saccharides was obtained. This did not appear to be due to the presence of a glycosulphatase in the enzyme preparation, since D-glucose 6-*O*-sulphate was not hydrolysed. Recent results obtained by D'Abramo & Lipmann (1957) suggest that in the formation of chondroitin sulphate, oligo- or poly-saccharide acceptors engage with 'active sulphate'.

Circumstantial evidence indicates that the same mechanism of sulphate activation may be involved in the biosynthesis of aryl sulphates as in that of sulphated polysaccharides. In cartilaginous tissue (Layton & Frankel, 1951; Loewi & Kent, 1957), and in the small intestine (Werner, 1953; Williams, 1947) formation of both types of sulphated product has been observed. The biosynthesis of D-glucose sulphate by enzymes from the partly autolysed mucous glands of *Charonia lampas* has been demonstrated by Suzuki, Takahashi & Egami (1957). Here, *p*-nitrophenyl [^{35}S]sulphate functioned as a donor (in contrast to adenosine 3′-phosphate 5′-phosphosulphate, cf. Gregory & Nose, 1957), and the results suggest the participation of transulphatases.

The soluble fraction from the colonic mucosa contains a number of enzymes besides the system which activates sulphate. Such enzyme preparations catalyse the breakdown of ATP to inosine, inosine monophosphate and hypoxanthine.

SUMMARY

1. Supernatant enzyme fractions from sheep colonic mucosa catalyse the formation of an 'active sulphate' from adenosine triphosphate and SO_4^{2-} ions.

2. The 'active sulphate' has been isolated and found to be an adenine nucleotide carrying a very labile sulphate ester. The compound is identical with or closely similar to adenosine 3′-phosphate 5′-phosphosulphate.

3. The 'active sulphate' acts as donor in the formation of phenolic sulphates by liver or mucosal enzymes. No evidence for the sulphation of simple mono- and di-saccharides could be obtained.

4. Mucosal enzymes catalyse breakdown of adenosine triphosphate to inosine, inosine monophosphate and hypoxanthine.

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The Structure of the Naturally Occurring Phosphoglycerides

4. ACTION OF CABBAGE-LEAF PHOSPHOLIPASE *D* ON OVOLECITHIN AND RELATED SUBSTANCES*

BY FLORA M. DAVIDSON† AND C. LONG‡
Department of Biological Chemistry, University of Aberdeen

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The enzyme phospholipase *D*, which liberates choline from the naturally occurring lecithins, has been found in several plants, including carrot root, cabbage, spinach and sugar-beet leaves, the latex of *Hevea brasiliensis* and defatted cottonseed meal (Hanahan & Chaikoff, 1947*a*, *b*, 1948; Smith, 1954; Kates, 1954; Tookey & Balls, 1956). In those plants investigated by Kates (1954), the enzyme was reported to reside exclusively in the plastids, and its activity was greatly increased by the addition of ether.

In the work to be described, an examination of several plant species for phospholipase *D* activity revealed that the leaves of Savoy cabbage and Brussels sprouts were much the richest sources. Furthermore, the enzyme was present in the cell sap as well as in the plastids. The enzyme from the cell sap has been partially purified and found to exhibit an absolute requirement for calcium ions. This enzyme preparation, which removes all the choline from ovolecithin and from synthetic L-α- and DL-α-lecithins, also acts upon egg phosphatidylethanolamine.

Evidence has been obtained for the presence of another enzyme in the supernatant from a Savoy-cabbage homogenate; this appears to be a phosphodiesterase, different from phospholipase *D*, which hydrolyses water-soluble phosphodiesters.

* Part 3: Long & Penny (1957).

† Present address: Dept. of Pathology, Institute of Orthopaedics, Royal National Orthopaedic Hospital, Stanmore, Middlesex.

‡ Present address: Physiology Department, Institute of Basic Medical Sciences, Royal College of Surgeons of England, London, W.C. 2.

Some of the results obtained with Savoy-cabbage phospholipase *D* have already been reported briefly (Davidson, Long & Penny, 1956; Davidson & Long, 1957).

EXPERIMENTAL

Materials

Ovolecithin. This was obtained from egg yolk and purified either through the cadmium complex (Pangborn, 1951) or by chromatography on silicic acid (Lea, Rhodes & Stoll, 1955). Some samples were hydrogenated catalytically as described by Long & Maguire (1954).

Dipalmitoyl-L-α-lecithin. This was a gift from Dr D. J. Hanahan.

Lysolecithin. A solution containing 600 mg. of ovolecithin in 400 ml. of dry peroxide-free ether was treated with 4 mg. of dried cottonmouth-moccasin venom (Ross Allen's Reptile Institute, Florida, U.S.A.) dissolved in 4 ml. of 0·005M-$CaCl_2$ (Long & Penny, 1957). The mixture was stirred mechanically at 18°. After 3 hr., the precipitated lysolecithin was separated by centrifuging, washed with 100 ml. of acetone and dissolved in 10 ml. of $CHCl_3$. After filtering off a trace of insoluble material, the filtrate was treated with 100 ml. of acetone. The precipitate of lysolecithin was finally dissolved in 20 ml. of $CHCl_3$ and stored at 2°. The ratio, equivalents of fatty acid ester: atoms of P, was 1·02.

Egg phosphatidylethanolamine. This was prepared from egg yolk by chromatography on silicic acid (Lea *et al.* 1955).

Ox-brain 'kephalin'. Material corresponding with fractions III–V of ox-brain kephalin was prepared by the method of Folch (1942). This consists largely of phosphatidylethanolamine and phosphatidylserine, together with the corresponding plasmalogens.

Sphingomyelin. A sample was given by Drs J. A. Lovern and J. Olley.

Synthetic phosphoglycerides. Dipalmitoyl-DL-α-lecithin, distearoyl-DL-α-lecithin, distearoyl-β-lecithin, dimyristoyl-DL-α-phosphatidylethanolamine and dimyristoyl-β-phosphatidylethanolamine were gifts from Drs T. Malkin and T. H. Bevan. A sample of dimyristoyl-L-α-lecithin was given by Dr E. Baer.

Glycerylphosphorylcholine. This was prepared from ovolecithin by the mild alkaline-hydrolysis procedure of Dawson (1954).

Sodium diphenyl phosphate. A specimen of pure diphenyl hydrogen phosphate was given by Dr T. Hofman. Another sample, given by L. Light and Co. Ltd., Colnbrook, Bucks, was converted into the sodium salt as follows. A solution of the material (10 g.) in ethanol (50 ml.) was neutralized with 40% (w/v) NaOH. Ether (250 ml.) was then added and the colourless crystalline ppt. of the sodium salt was filtered off. It was used without further purification.

Di-p-nitrophenyl hydrogen phosphate. A pure specimen was given by Dr T. Hofman.

Phosphorylcholine. A sample of the calcium salt was given by Dr M. G. Macfarlane.

Choline iodide. This was prepared from methyl iodide and dimethylaminoethanol, and was used as a choline standard, since it is non-deliquescent (personal communication from Dr T. H. Bevan).

Calcium phosphate gel. This was prepared by the procedure of Keilin & Hartree (1951). The suspension had a dry weight of 15 mg./ml.

Buffers. For experiments carried out at pH 5·6, a sodium acetate–acetic acid buffer was used. For other pH values, equal volumes of 0·25M-solutions of acetic acid and 2-amino-2-hydroxymethylpropane-1:3-diol (tris) were mixed, the pH was then adjusted to the desired value by the addition of HCl or NaOH, and the buffer solution was diluted so that the final concentrations of acetate and tris were each 0·1M.

All other materials were the best commercial preparations.

Sources of enzyme. Most of the vegetables were purchased locally. Onion roots were grown by placing onion sets on the necks of 100 ml. conical flasks completely filled with tap water, and leaving for one month.

Methods

Analytical procedures. Dry weights were obtained by drying overnight at 105°. Protein N was determined on precipitates insoluble in 5% (w/v) trichloroacetic acid by a micro-Kjeldahl method. Choline was determined by the enneaiodide procedure described by Shapiro (1953), ethanolamine by the 1-fluoro-2:4-dinitrobenzene method of Axelrod, Reichenthal & Brodie (1953), or by the periodate procedure of Burmaster (1946), and serine by the method of Axelrod *et al.* (1953). Phenol was determined by means of the reagent of Folin & Ciocalteu (1927), and phosphate by the method of Berenblum & Chain (1938) as modified by Long (1943). Fatty acid ester was determined by the hydroxamic acid procedure of Shapiro (1953). After ashing at 650° and solution of the residue in HCl, followed by neutralizing, calcium was determined according to Clark & Collip (1925).

Enzyme preparations. The plant material, sometimes after a preliminary mincing, was homogenized with 2·5–10 vol. of water, usually for 5 min. in a top-drive macerator (Townson and Mercer Ltd., Croydon), though other machines were also used with similar results. The fibrous material was removed by filtering through silk. The suspension was then centrifuged for 30 min. at 13 000 *g* in a refrigerated centrifuge (Angle 13; Measuring and Scientific Equipment Co. Ltd.). Occasionally the supernatant and sediment were tested separately for phospholipase *D* activity, but normally only the supernatant was examined. In most experiments Savoy cabbage (*Brassica oleracea* var. *Bullata*) was used; when this was not available, other cabbages or Brussels sprouts were used according to the season.

Reaction system for measuring phospholipase D *activity.* The enzymic degradation was carried out in 15 ml. glass-stoppered test tubes. To 1·25 ml. of an emulsion of ovolecithin (16 μmoles) in 0·1M-acetate buffer, pH 5·6, 0·25 ml. of M-$CaCl_2$ and 1 ml. of enzyme preparation were added. The tubes were warmed to 26°. At zero time 1 ml. of ether was added, the contents of the tubes were shaken vigorously and the reaction mixtures were incubated at 26° for 10 or 12 min. The enzymic reaction was stopped by addition of 1 ml. of 30% (w/v) trichloroacetic acid. After adding 1·5 ml. of water, the mixtures were again shaken and the tubes were centrifuged. The ethereal phase, containing most of the phosphatidic acid and unchanged ovolecithin, was removed with a Pasteur pipette and discarded. The

aqueous phase was filtered and the clear filtrate was warmed on a water bath at 50° to remove dissolved ether; this last step was essential since ether interferes with the subsequent estimation of choline. Choline was determined on 2 ml. of the ether-free filtrate.

With substrates other than ovolecithin, the general procedure was similar. However, 0·1 ml. of $CHCl_3$ was often added to the reaction mixture, as well as 1 ml. of ether, especially with those substrates which were sparingly soluble in ether. Parallel experiments with ovolecithin as substrate showed that $CHCl_3$ had no inhibitory effect on phospholipase *D* activity, but rather produced a slight activation.

Relative chlorophyll content. Samples (1 ml.) of the Savoy-cabbage-leaf fractions were extracted with 85% (v/v) acetone. The combined filtrates and washings were diluted to 10 ml. and the optical densities were determined in a photo-electric colorimeter (Evans Electroselenium Ltd.) with a red filter. This is essentially the procedure of Clendenning & Gorham (1950).

RESULTS

Distribution of phospholipase D *activity in plants*

Phospholipase *D* activity was assayed by determining the quantity of choline liberated from ovolecithin by an homogenate of the plant tissue. The experimental conditions were as described in the Methods section. As shown in Table 1, of the limited number of species examined, Savoy cabbage had the highest activity and onion the lowest. None of the plant tissues tested was completely lacking in phospholipase *D* activity. Reliable results could not be obtained with potato tuber, since the presence of starch interfered with the determination of choline by the enneaiodide method.

A small amount of acid-soluble organic phosphate was liberated from ovolecithin by homogenates of the leaves and tubers of potatoes (0·04 and 0·31 μg.atoms of P/mg. dry wt./hr. respectively); a trace of inorganic phosphate was formed by the action of an onion homogenate. Other plant homogenates did not liberate acid-soluble phosphorus from ovolecithin under the conditions used for determining phospholipase *D* activity.

Enzymic degradation of ovolecithin by Savoy-cabbage-leaf supernatant

Savoy-cabbage homogenate showed an average phospholipase *D* activity of 2·51 μmoles of choline/mg. dry wt./hr. (Table 1), which corresponds to a $Q_{choline}$ value (μl. of choline/mg. dry wt./hr.) of about 56. Control experiments showed that when the homogenate was incubated with choline chloride, none of the latter was destroyed. With the supernatant from a Savoy-cabbage homogenate, the average rate of liberation of choline from ovolecithin was 4·2 μmoles/mg. dry wt./hr. (average $Q_{choline}$, 94). In these experiments, there was no liberation of choline in the absence of ovolecithin, but the Savoy cabbage contained a small amount of material which was estimated as choline; this blank value has been deducted in all cases.

In the absence of ether, the rate of enzymic degradation during a 10 min. incubation period was too low to be measurable, so that it was permissible, and very convenient, to take zero time as the moment of adding the ether. Variations in the volume of ether used affected somewhat the rate of liberation of choline. In one experiment, 5·5 and 8·2 μmoles of choline were liberated in the presence of 0·5 and 3·0 ml. of ether respectively.

The rate of hydrolysis was practically constant during the first 20 min. of incubation, provided that less than 50% of the 16 μmoles of ovolecithin originally present was hydrolysed; thereafter, the rate declined owing to depletion of substrate. With

Table 1. *Distribution of phospholipase* D *activity in plants*

Conditions for determining phospholipase *D* activity are described in the Methods section; the less active plant tissues were incubated for longer periods. Whole homogenates were used.

Plant	Portion used	No. of observations	Fresh tissue (mg.)	Duration (min.)	Average phospholipase *D* activity (μmoles of choline/mg. dry wt./hr.)
Savoy cabbage	Leaves	3	100	10	2·51
Other cabbages	Leaves	2	100	10	0·80
Brussels sprouts	Sprouts	1	100	10	2·18
Turnip	Leaves	1	400	10	0·58
Turnip	Midribs of leaves	1	400	10	0·34
Lettuce	Leaves	2	400	10	0·54
Carrot	Root	1	400	10	0·44
Potato	Leaves	1	400	10	0·19
Beetroot	Globe	2	400	10	0·11
Pea	Dried seeds	1	100	10	0·09
Parsley	Leaves	1	333	60	0·05
Onion	Roots	1	200	180	0·03
Onion	Bulb	1	800	120	0·01

4·5 μmoles of ovolecithin, enzymic hydrolysis was complete within 2 hr.

Kates (1954) reported an optimum pH between 5 and 6 for phospholipase *D* in a suspension of cabbage-leaf chloroplasts. A similar value has been found with the supernatant from a Savoy-cabbage homogenate.

Activation by calcium ions. In the absence of added Ca^{2+} ions, the rate of enzymic hydrolysis of ovolecithin by Savoy-cabbage supernatant was very low. In the system containing 16 μmoles of ovolecithin, the maximum rate was observed in the presence of 0·1 M-$CaCl_2$ (Davidson *et al.* 1956), and this was about seven times the rate observed in the absence of added Ca^{2+} ions. The optimum concentration of Ca^{2+} ions was independent of the enzyme concentration, but was roughly proportional to the amount of ovolecithin used as substrate; a similar relationship had been found for snake-venom phospholipase *A* by Long & Penny (1957). For phospholipase *D*, the optimum ratio was 15 μmoles of Ca^{2+} ions/μmole of ovolecithin, whereas for phospholipase *A* Long & Penny (1957) found a value of about 0·03.

Strontium and Ba^{2+} ions also activated phospholipase *D*. At a final concentration of 0·1 M, $SrCl_2$ and $BaCl_2$ showed 67 and 34 % respectively of the activity of 0·1 M-$CaCl_2$ (Davidson *et al.* 1956). A smaller activation (12 % of the Ca^{2+} ion effect) was shown by 0·1 M-$ZnCl_2$. The following metal chlorides (0·2 M with respect to Cl^- ion) were either without effect or slightly inhibitory: NaCl, KCl, NH_4Cl, $MgCl_2$, $MnCl_2$ and $FeCl_3$.

Action of Savoy-cabbage supernatant on other substrates

The Savoy-cabbage supernatant liberated all the choline from the following lecithins: ovolecithin, hydrogenated ovolecithin, dipalmitoyl-L-α-lecithin, lysolecithin, synthetic dimyristoyl-L-α-lecithin, dipalmitoyl-DL-α-lecithin and distearoyl-DL-α-lecithin. Synthetic distearoyl-β-lecithin was only slowly attacked, as was sphingomyelin. In those instances where the effects were examined, enzymic degradation was stimulated by Ca^{2+} ions and by ether, with the exception that the hydrolysis of lysolecithin was inhibited by ether. This observation may be associated with the well-known water-solubility and ether-insolubility of lysolecithin. The rate of hydrolysis of lysolecithin was 0·05–0·12 of that of ovolecithin.

Ethanolamine was readily liberated from egg phosphatidylethanolamine and from synthetic dimyristoyl-DL-α-phosphatidylethanolamine, but dimyristoyl-β-phosphatidylethanolamine was acted upon less rapidly. Some of these results have been summarized by Davidson *et al.* (1956). As with ovolecithin, Ca^{2+} ions were necessary for the enzymic degradation of phosphatidylethanolamine. The optimum pH with this substrate was between 5·6 and 6·0. The supernatant from Savoy cabbage attacked egg phosphatidylethanolamine at about 60 % of the rate with ovolecithin; with the supernatant from Brussels sprouts, the two substrates were hydrolysed at approximately equal rates.

Savoy-cabbage-leaf supernatant slowly hydrolysed the water-soluble substrates sodium diphenyl phosphate and glycerylphosphorylcholine, but the rates were increased only about 20–40 % in the presence of Ca^{2+} ions. The rate of hydrolysis of neither substrate was increased by the presence of ether. The optimum pH was significantly higher for diphenyl phosphate (about 7) and for glycerylphosphorylcholine (about 8) than for ovolecithin or egg phosphatidylethanolamine. Under optimum conditions, the rates of hydrolysis of diphenyl phosphate and glycerylphosphorylcholine by a Savoy-cabbage supernatant were about 2 and 1 % respectively of the rate for ovolecithin.

Phosphorylcholine was hydrolysed even more slowly than was glycerylphosphorylcholine.

Distribution of phospholipase D *activity between soluble and insoluble cell components*

Kates (1954) has reported that phospholipase *D* activity is present in leaf chloroplasts. The distribution of the enzyme has now been determined in (*a*) the outer dark-green leaves, (*b*) the inner light-green leaves, and (*c*) the yellow heart of a Savoy cabbage. Weighed samples of leaves (15 g.) were ground in a mechanical mortar and then homogenized with 35 ml. of water for 5 min. in a top-drive macerator (Measuring and Scientific Equipment Co. Ltd.). Each sample was then treated in the following way. Phospholipase *D* activity and relative chlorophyll content were determined on measured volumes of the homogenate. The rest was filtered through silk and the enzymic activity of the fibre-free suspension was determined. The remainder of the suspension was centrifuged at 13 000 ***g*** for 30 min. at 10°, and the residue was suspended in a volume of water equal to that of the supernatant removed. Both the suspended residue and the almost clear supernatant were tested for phospholipase *D* activity. The results are shown in Table 2.

It will be seen that, whereas the insoluble fraction from each type of leaf had approximately the same phospholipase *D* activity when measured in the presence of 0·1 M-$CaCl_2$, the soluble fraction from the yellow heart had a much higher activity than the corresponding fraction from the outer green leaves. The activity of the supernatant from the inner green leaves was intermediate in magnitude. In the insoluble fraction from the outer green leaves, further investigation showed that the

Table 2. *Phospholipase* D *activity of soluble and insoluble cell components of aqueous Savoy-cabbage homogenates*

Experimental details are given in the text. Conditions for determining enzymic activity and relative chlorophyll content are described in the Methods section. Parallel determinations of enzymic activity were also made in the absence of added $CaCl_2$. Volume of suspension or supernatant used was 1 ml. Incubation was for 12 min. at 26°.

Type of leaf	Relative chlorophyll content of homogenate (galvanometer reading)	$CaCl_2$ concn. (M)	Phospholipase *D* activity (μmoles of choline liberated/12 min.) Whole homogenate	Fibre-free suspension	Soluble fraction	Insoluble fraction
(*a*) Dark-green outer	46	0	5·0	5·1	0·8	1·7
		0·1	8·8	9·8	4·1	4·3
(*b*) Light-green inner	19	0	2·5	2·0	0·4	0·5
		0·1	11·3	10·4	7·1	4·3
(*c*) Yellow 'heart'	0	0	2·7	2·8	0·5	0·5
		0·1	10·2	10·5	11·7	4·1

enzyme was very tightly bound to the particles. Repeated washing with water did not diminish the phospholipase *D* activity. The insoluble fraction, consisting of both intact and damaged chloroplasts, was further comminuted in a Potter–Elvehjem (1936) homogenizer, but the enzyme remained attached to the particles, which were composed of partly disintegrated grana.

It will also be noted that the insoluble fraction of the outer green leaves possessed an appreciable phospholipase *D* activity in the absence of added Ca^{2+} ions; this activity is equivalent to the liberation of 1·6 μmoles of choline/mg. dry wt./hr. and is consistent with the findings of Kates (1954), who observed the liberation of choline from ovolecithin by cabbage-leaf chloroplasts in the absence of added Ca^{2+} ions. His published data, when converted into μmoles of choline/mg. dry wt./hr., correspond with 2·2, 1·9 and 0·8 for sugar-beet, spinach and cabbage leaves respectively, values which are very similar to that found in the present work. These calculations suggest that, on the basis of dry weight, the plastid enzyme of Kates is only about 40% as active as the soluble phospholipase *D* studied here; however, the activity of the plastids can be increased considerably by addition of Ca^{2+} ions.

In the experiment shown in Table 2, the outer dark-green leaves gave rise to an insoluble particle fraction, which on analysis contained 9·8 μg. of calcium/mg. dry wt.; on the assumption that this calcium is ionized and is contained within the particles in a non-diffusible form, this amount would correspond with an internal Ca^{2+} ion concentration of about 0·04 M, taking the dry weight of the particles as 13% of the wet weight. At this Ca^{2+} ion concentration, the phospholipase *D* activity would be expected to be submaximum (Davidson *et al.* 1956), which is consistent with the observation that, in the presence of added Ca^{2+} ions, the activity is increased about 2·5-fold.

The requirement of phospholipase *D* for Ca^{2+} ions is supported by two further pieces of evidence, namely the finding by Kates (1954) that the plastid phospholipase *D* activity was inhibited by fluoride, and our own observation that 0·01 M-ethylenediaminetetra-acetate completely suppressed the phospholipase *D* activity of a whole homogenate of Savoy cabbage in the absence of added Ca^{2+} ions.

Partial purification of phospholipase D *from Savoy-cabbage supernatant*

The following procedure was found to result in a 45-fold purification of the enzyme, on the basis of protein N, with a recovery of 50% of the total activity. The inner light-green leaves of a Savoy cabbage (200 g.) were homogenized with 300 ml. of water for 5 min. The homogenate was largely freed from fibre by squeezing through silk and then centrifuged at 13 000 *g* for 30 min., giving about 380 ml. of almost clear supernatant (stage 1). The supernatant was maintained at 55° for 5 min. and then rapidly cooled. The bulky precipitate which had formed was centrifuged down as before and discarded; the supernatant amounted to 340 ml. (stage 2). This supernatant was cooled to 0° and then treated with 2 vol. of acetone at −15°; after 10 min., the precipitate, which contained most of the enzyme, was centrifuged down (stage 3). The precipitate was dissolved in 80 ml. of water, and a slight amount of insoluble material was removed by centrifuging at 13 000 *g*. A sample (12 ml.) of the clear solution was then treated with 7·2 ml. of calcium phosphate gel (15 mg. dry wt./ml.). The enzyme was completely adsorbed. (The amount of gel required to adsorb the enzyme varied between individual experiments, and had to be determined in each case.) The gel was centrifuged down at 0° at 5000 *g* for 20 min. The enzyme was eluted from the gel with 12 ml. of 0·05 M-phosphate buffer (Sørensen), pH 7·5, and the eluate was dialysed against water for 2 hr. at 2°. The enzyme was again

Table 3. *Partial purification of Savoy-cabbage phospholipase* D

The fractionation procedure is described in the text. Determinations of dry weight, protein N and phospholipase *D* activity (with ovolecithin as substrate) are as given in the Methods section. All concentrations and activities are calculated as if the various fractions had been diluted to the original volume of the crude supernatant.

Stage	Treatment	Dry wt. (mg./ml.)	Protein N (mg./ml.)	Phospholipase *D* activity (μmoles of choline/ml./ 10 min.)	Phospholipase *D* activity (μmoles of choline/mg. of protein N/ 10 min.)	Purification	Yield (%)
1	Crude supernatant	21·5	0·378	7·4	19·6	1	100
2	Supernatant after heat-coagulation	19·6	0·153	7·4	48·4	2·5	100
3	Pptd. with 2 vol. of acetone	1·3	0·048	5·7	119	6·1	77
4	Eluate from $Ca_3(PO_4)_2$ gel	—	0·0042	3·8	905	46·2	51

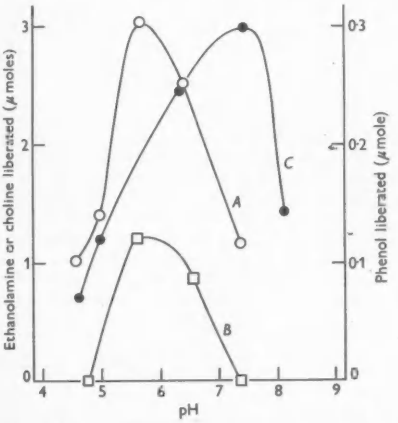

Fig. 1. pH–Activity curves for enzyme preparation from Savoy cabbage. Reaction mixtures contained: partially purified (stage 3) enzyme (1 ml.), 0·1 M-acetate–tris buffer (1 ml.), M-$CaCl_2$ (0·25 ml.) and substrate; total aqueous volume 2·5 ml. Substrates: curve *A*, ovolecithin (16 μmoles); curve *B*, egg phosphatidylethanolamine (6·2 μmoles); curve *C*, sodium diphenyl phosphate (15 μmoles). Curve *A*, ether (1 ml.) added at zero time; curve *B*, ether (1 ml.) + $CHCl_3$ (0·1 ml.) added at zero time. Incubation at 26° for 10 min. (curve *A*), 12 min. (curve *B*) or 30 min. (curve *C*).

precipitated with 2 vol. of acetone at 0°. It was finally dissolved in 12 ml. of water and stored at −15° (stage 4). At each stage, dry weight, protein N and phospholipase *D* activity were determined. The results are summarized in Table 3.

Properties of partially purified phospholipase D

The phospholipase *D* preparation of highest purity (Table 3; stage 4) proved to be rather unstable even when stored as a frozen solution at −15°. For this reason routine purification has not been continued beyond the acetone-precipitation step (Table 3; stage 3). This partially purified enzyme acted readily upon ovolecithin and egg phosphatidylethanolamine. The optimum pH was the same as for the crude Savoy-cabbage supernatant, namely about 5·6 (Fig. 1). Little or no activity was observed in the absence of added Ca^{2+} ions (Table 4) or of ether.

Table 4. *Effect of calcium ions on the degradation of ovolecithin and egg phosphatidylethanolamine by partially purified phospholipase* D

Enzyme: partially purified (Table 3; stage 3) preparation from Savoy-cabbage supernatant. Substrates: ovolecithin, 4·8 μmoles in Expt. 1; egg phosphatidylethanolamine, 6·2 μmoles in Expt. 2. Other conditions were similar to those in Fig. 1, except that acetate buffer, pH 5·6, was present in all reaction mixtures, and the concentration of $CaCl_2$ was varied.

$CaCl_2$ concn. (M)	Expt. 1 Choline formed (μmoles/12 min.)	Expt. 2 Ethanolamine formed (μmoles/30 min.)
0	0	0·21
0·01	1·55	—
0·10	1·75	1·69
1·00	0·75	—

A comparison was made of the rates of hydrolysis of some phosphoglycerides of known structure by the partially purified enzyme. Taking the rate of hydrolysis of ovolecithin as 100, the relative rates were: dimyristoyl-L-α-lecithin, 100; dipalmitoyl-L-α-lecithin, 85; dipalmitoyl-DL-α-lecithin, 15; distearoyl-DL-α-lecithin, 2; dipalmitoyl-β-lecithin, 2; distearoyl-β-lecithin, 1; egg phosphatidylethanolamine, 60; dimyristoyl-DL-α-phosphatidylethanolamine, 4; dimyristoyl-β-phosphatidylethanolamine, 0·3. Prolonged incubation of all these substrates, except dimyristoyl-β-phosphatidylethanolamine, resulted in virtually complete liberation of the base.

When fractions III–V of ox-brain kephalin Folch, 1942), which mainly consist of phosphatidylethanolamine and phosphatidylserine to-

gether with the corresponding plasmalogens, were acted upon by the partially purified enzyme, the ethanolamine was released much more rapidly than the serine. The ethanolamine was ultimately released completely, but only 63 % of the serine was released even after incubation for 24 hr.

The partially purified enzyme preparation still acted upon diphenyl phosphate at about 2 % of the rate found with ovolecithin, but had very little effect on glycerylphosphorylcholine (about 0·2 % of the rate with ovolecithin). That the hydrolysis of diphenyl phosphate is catalysed by an enzyme different from phospholipase *D* is clearly indicated by the experiments shown in Table 5. Expt. 1 shows the extent of adsorption of each enzyme when treated with different amounts of calcium phosphate gel. In Expt. 2 a partial separation of the two enzymes was achieved by treatment with successive amounts of gel followed by elution with phosphate.

Enzymic degradation of ovolecithin in the presence of water-soluble phosphodiesters

Diphenyl phosphate. Since the results obtained in Table 5 clearly showed that the enzymic hydrolyses of ovolecithin and of diphenyl phosphate were catalysed by separate enzymes, it was to be expected that mixed-substrate experiments would show that ovolecithin and diphenyl phosphate were degraded independently of one another. However, as shown in Table 6, diphenyl phosphate was found to stimulate the enzymic hydrolysis of ovolecithin. The effect increased with increasing concentrations of diphenyl phosphate up to about 0·16 M. Di-*p*-nitrophenyl phosphate had a similar though quantitatively smaller effect.

The possibility that the stimulation of phospholipase *D* activity might be due to the products of enzymic hydrolysis of diphenyl phosphate, rather than to diphenyl phosphate itself, was investigated by examining the action of monophenyl phosphate, phenol and inorganic phosphate on the system. As shown by the experiment in Fig. 2, in which a crude supernatant from a Savoy-cabbage homogenate was used, all these compounds had a slight stimulating effect at low concentrations, the effect always being much less than that produced by the corresponding concentration of diphenyl phosphate. Phenol was the most active; monophenyl phosphate and inorganic phosphate inhibited at higher concentrations. It is quite impossible therefore that diphenyl phosphate exerted its stimulatory effect after being enzymically hydrolysed. This conclusion was supported by the observation that diphenyl phosphate exerted an almost identical effect on the hydrolysis of ovolecithin by a phospholipase *D* preparation from which the phosphodiesterase activity had been removed by

Table 5. *Separation of phospholipase* D *and phosphodiesterase activities*

Expt. 1. Enzyme solution (Table 3, stage 3; 2 ml.) was treated with different volumes of $Ca_3(PO_4)_2$ gel; water was added to final volume 3·5 ml. After centrifuging, the supernatants were tested for phospholipase *D* and phosphodiesterase activities. Phospholipase *D* activity was determined after incubation for 12 min. with ovolecithin (16 μmoles) as substrate, at pH 5·6 and in the presence of 0·1 M-$CaCl_2$ and ether (1 ml.). Phosphodiesterase activity was determined after incubation with 15 μmoles of diphenyl phosphate at pH 6·6, in the presence of 0·1 M-$CaCl_2$ and in the absence of ether.

Vol. of $Ca_3(PO_4)_2$ gel (ml.)	Phospholipase *D* activity (μmoles of choline/ml./hr.)	Phosphodiesterase activity (μmoles of phenol/ml./hr.)
0	44·4	0·718
0·3	46·6	0·434
0·6	40·0	0
0·9	28·6	0
1·2	26·6	0
1·5	24·0	0

Expt. 2. Same enzyme solution was treated with 0·6 ml. of $Ca_3(PO_4)_2$ gel and after 15 min. the suspension was centrifuged. The ppt. was eluted with 2 ml. of 0·1 M-phosphate buffer, pH 7·5, and the eluate was dialysed (fraction 1). The supernatant was treated with 1·4 ml. of gel; after centrifuging, this ppt. was also eluted with phosphate and the eluate was dialysed (fraction 2). All activities relate to 0·5 ml. of the untreated enzyme or to fractions derived therefrom. Activities were determined as in Expt. 1.

	Phospholipase *D* activity (μmoles of choline/hr.) (*a*)	Phosphodiesterase activity (μmoles of phenol/hr.) (*b*)	Ratio (*a*/*b*)
Untreated enzyme	22·2	0·36	62
Fraction 1	3·7	0·17	21
Fraction 2	15·6	0·015	~1000

Table 6. *Effect of diphenyl phosphate on the activity of partially purified phospholipase* D

Reaction mixtures contained the following components: ovolecithin, 16 μmoles; partially purified enzyme (stage 3) from Savoy cabbage, 1 ml.; acetate buffer (pH 5·6), 1·25 ml.; M-$CaCl_2$, 0·25 ml.; total aqueous volume 3 ml. Ether (1 ml.) was added at zero time. Incubation was for 10 min. at 26°. Under these conditions, 3·8 μmoles of choline were liberated in the absence of diphenyl phosphate.

Diphenyl phosphate concn. (M)	Phospholipase *D* activity (% of control)	
	Expt. 1	Expt. 2
0	100	100
0·004	157	—
0·012	187	—
0·040	236	197
0·080	—	208
0·160	—	230
0·240	—	210
0·400	—	29

adsorption on calcium phosphate gel. At a concentration of 0·02 M-diphenyl phosphate, activations of the order of 170–250 % were obtained at values between pH 5·0 and 7·4 and with $CaCl_2$ concentrations between 0·025 and 0·25 M. The percentage activation was not affected by the degree of purification of the enzyme used. At the highest concentration used (0·4 M), diphenyl phosphate had an inhibitory effect (Table 6), which was probably due to its calcium-binding activity.

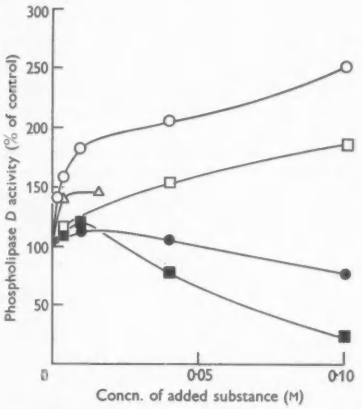

Fig. 2. Effects of diphenyl phosphate (○), monophenyl phosphate (●), di-*p*-nitrophenyl phosphate (△), phenol (□) and inorganic phosphate (■) on the rate of hydrolysis of ovolecithin by phospholipase *D* of Savoy-cabbage supernatant. The reaction mixtures contained: ovolecithin, 16 μmoles; M-$CaCl_2$, 0·25 ml.; 0·1 M-acetate buffer (pH 5·7), 1·25 ml.; additions, as indicated; total aqueous volume 2·5 ml. Ether (1 ml.) was added at zero time. Incubation was for 12 min. at 26°.

Table 7. *Activation of partially purified phospholipase* D *by glycerylphosphorylcholine*

Partially purified enzyme (stage 3) from Brussels sprouts was used. Experimental conditions were as described in the Methods section. Substrates: Expt. 1, ovolecithin (15 μmoles); Expt. 2, egg phosphatidylethanolamine (6·4 μmoles). Amount of glycerylphosphorylcholine (GPC) as indicated. Incubation for 10 min. at 26°.

	Expt. 1 (μmoles of choline liberated)	Expt. 2 (μmoles of ethanolamine liberated)
GPC (7·8 μmoles)	0	—
Ovolecithin	3·86	—
Ovolecithin + GPC (7·8 μmoles)	5·50	—
GPC (13·2 μmoles)	—	0
Egg phosphatidylethanolamine	—	2·93
Egg phosphatidylethanolamine + GPC (13·2 μmoles)	—	3·48

The rates of enzymic degradation of phosphatidylethanolamine, dipalmitoyl-β-lecithin and lysolecithin were also found to be stimulated by diphenyl phosphate.

Glycerylphosphorylcholine. Glycerylphosphorylcholine was also found to stimulate the enzymic breakdown of both ovolecithin and phosphatidylethanolamine. Results of an experiment with the partially purified enzyme from Brussels sprouts are shown in Table 7. After the incubation of the enzyme with glycerylphosphorylcholine both in the presence and absence of ovolecithin, the filtrates, free from protein and lipid, were hydrolysed by heating with N-HCl on a boiling-water bath for 20 min., in order to liberate choline from the added glycerylphosphorylcholine. In both cases, the amount of extra choline formed corresponded closely with the amount expected on the assumption that no glycerylphosphorylcholine had undergone enzymic hydrolysis. This result shows that the glycerylphosphorylcholine caused activation without being hydrolysed.

When egg phosphatidylethanolamine was used as substrate, no choline was liberated from the added glycerylphosphorylcholine.

DISCUSSION

Since phospholipase *D* is particularly active in the leaves of Savoy cabbage, this plant has been used for most of the work described. It has not been determined whether the properties of the enzyme from other less potent sources are similar to those from Savoy cabbage, although from observations made with the limited number of plants investigated we have no reason to suspect any important differences.

Although Kates (1954) concluded that phospholipase *D* was present exclusively in the plastids, we have readily obtained it in solution, especially from the 'heart' of Savoy cabbage. The probable explanation for the discrepancy between our results and those of Kates (1954) is that the plastids contain sufficient calcium to show a measurable activity, whereas the concentration of Ca^{2+} ions of the supernatants is so low that activity can only be demonstrated in the presence of added Ca^{2+} ions.

There are several reasons for believing that the soluble enzyme did not arise in our experiments from the plastids during the homogenizing procedure. (1) The plastids of Savoy cabbage are known to be very resistant to disruption. (2) Even after the plastids had been ground in a Potter–Elvehjem homogenizer, the enzyme remained firmly bound to the grana, none passing into solution. (3) The enzymic activity of the insoluble particles derived from the outer green leaves of Savoy cabbage, when measured in the absence of

added Ca^{2+} ions, was found to be similar to those reported by Kates (1954); this would not have been the case if enzyme had been leached from the particles during the preparation of the homogenate. (4) The activity of whole homogenates (particles plus supernatant), when measured in the presence of Ca^{2+} ions, greatly exceeded that found in plastids alone by Kates (1954).

Like the plastid enzyme, the soluble enzyme was also found to require the addition of ether for activity to become measurable.

The partial purification of the enzyme followed conventional lines. However, the fraction of highest purity proved to be rather labile, so that the product obtained after selective heat-denaturation and acetone-precipitation, which proved fairly stable when stored as a frozen solution, was normally used. This enzyme preparation was almost inactive in the absence of added Ca^{2+} ions, a fact which had earlier been found with the crude soluble enzyme (Davidson *et al.* 1956).

This partially purified enzyme acted readily upon both ovolecithin and egg phosphatidylethanolamine. Its action upon DL-α- and -β-lecithins showed clearly that this enzyme was not specific for substrates possessing the L-α-configuration; in this respect it differs from the phospholipase *A* of snake venom (Long & Penny, 1957). The partially purified enzyme also had a slight action upon diphenyl phosphate. However, it was possible, by selective adsorption upon calcium phosphate gel, to separate the enzymes acting upon phosphoglycerides from those acting upon water-soluble phosphodiesters.

The activation of phospholipase *D* by water-soluble phosphodiesters such as diphenyl phosphate and glycerylphosphorylcholine was unexpected. These activators exerted their effects with all those substrates of phospholipase *D* which were tested, and there was good evidence to show that the activation process was not accompanied by the enzymic hydrolysis of the activators. We are unable to offer any explanation for this activation.

SUMMARY

1. Evidence has been obtained that a soluble phospholipase *D* is present in the leaves of Savoy cabbage and in several other plants, in addition to the enzyme of plastids studied by Kates (1954).

2. The soluble enzyme, which is found in highest amount in the yellow 'heart' of Savoy cabbage, has been partially purified. It acts readily upon ovolecithin, egg phosphatidylethanolamine and other phosphoglycerides possessing the L-α structure; it has a smaller activity towards synthetic substrates with the DL-α or -β structure. In all cases the enzyme required the presence of both ether and calcium ions. Lysolecithin also underwent slow enzymic hydrolysis, a process which required calcium ions but was inhibited by ether.

3. Diphenyl phosphate and other water-soluble phosphodiesters were slightly hydrolysed both by a crude Savoy-cabbage supernatant and by the partially purified enzyme. It was possible to separate the enzyme acting upon phosphoglycerides (phospholipase *D*) from that acting on water-soluble phosphodiesters (phosphodiesterase) by selective adsorption upon calcium phosphate gel.

4. The partially purified phospholipase *D* was stimulated by diphenyl phosphate, di-*p*-nitrophenyl phosphate and glycerylphosphorylcholine.

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Inhibition of Glycosidases by Aldonolactones of Corresponding Configuration

2. INHIBITORS OF β-*N*-ACETYLGLUCOSAMINIDASE*

By JUNE FINDLAY, G. A. LEVVY and C. A. MARSH
Rowett Research Institute, Bucksburn, Aberdeenshire

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Conchie & Levvy (1957) found that, in general, the enzymic hydrolysis of a simple glycoside was inhibited by the aldonolactone corresponding in configuration to the sugar residue of the substrate. This suggested that the structural requirements necessary for a specific inhibitor of β-*N*-acetylglucosaminidase would be fulfilled by the compound 2-acetamido-2-deoxygluconolactone (*N*-acetylglucosaminonolactone). Hitherto the most effective competitive inhibitor of this enzyme has been the hydrolysis product, *N*-acetylglucosamine, which has a considerably lower affinity than the substrates customarily used for assay (Pugh, Leaback & Walker, 1957*b*).

N-Acetylglucosaminonolactone has not previously been synthesized, although Hope & Kent (1955) stated that the crude product of the treatment of glucosaminic acid with keten had the characteristics of a lactone. Partial acetylation of glucosaminic acid with acetic anhydride yielded a product, which, while not crystalline, possessed the properties of the required compound. Another obvious route was by selective oxidation of *N*-acetylglucosamine, and this was performed successfully by two different methods. As was expected, *N*-acetylglucosaminonolactone proved a potent inhibitor of β-*N*-acetylglucosaminidase.

Ram testis and emulsin preparations with β-*N*-acetylglucosaminidase activity have been shown to hydrolyse *p*-nitrophenyl *N*-acetyl-β-D-galactosaminide, and it has been tentatively concluded that the glucosaminidase is responsible (Heyworth, Borooah & Leaback, 1957). If this is so, 2-acetamido-2-deoxygalactonolactone (*N*-acetylgalactosaminonolactone) would also be expected to inhibit the enzymic hydrolysis of *N*-acetyl-β-D-glucosaminides; this has been found to occur. The complex structural changes in the inhibitory lactones under varying conditions of pH have been studied, largely by changes in their inhibitory power.

For most experiments, preparations of adult rat epididymis were used, since this tissue was found by Conchie, Findlay & Levvy (1956) to have much greater β-*N*-acetylglucosaminidase activity than any other studied: the action of the inhibitors on mammalian α-*N*-acetylglucosaminidase was also examined. The visceral hump of the common limpet (*Patella vulgata*) has been shown (Conchie & Levvy, 1957) to be a rich source of both α- and β-*N*-acetylglucosaminidase activity, and the effects of the lactones on the limpet enzymes have also been studied.

* The paper by Conchie & Levvy (1957) is to be regarded as Part 1.

EXPERIMENTAL

Analytical methods

Acetyl was determined by a modification of the method of Kuhn & Roth (1933).

Optical activity was measured on the Hilger Standard polarimeter, where possible with a 1 or 2 dm. tube; for measurements of $[\alpha]_D$ changes in *N*-acetylglucosaminonolactone solutions a 0·5 dm. microtube was employed.

All melting points are corrected. Measurement of pH was with the glass electrode.

Synthetic methods

Evaporations of solutions were done on the water bath under reduced pressure.

Phenyl N-*acetyl*-α- *and* -β-*glucosaminides.* These were prepared as described by Conchie & Levvy (1957). The α-anomer had m.p. 244–246°, $[\alpha]_D^{22}$ +210° in water (*c*, 1) and the β-anomer m.p. 248–249°, $[\alpha]_D^{22}$ −3° in water (*c*, 1·3). Roseman & Dorfman (1951) give values of m.p. 246–247°, $[\alpha]_D^{25}$ +213° and m.p. 249–250°, $[\alpha]_D^{25}$ −10·3° respectively.

p-*Nitrophenyl* N-*acetyl*-β-*glucosaminide.* By preliminary experiment it was found more convenient to use β-glucosamine penta-acetate, and not the usual equilibrium mixtures of the anomers, for condensation with *p*-nitrophenol; other modifications of the original procedure (Westphal & Schmidt, 1952) were necessary to obtain a satisfactory yield of the β-glycoside tetra-acetate. Our attention was drawn by Dr D. H. Leaback to the use of $ZnCl_2$, which usually favours the formation of α-D-sugar acetates (see Conchie, Levvy & Marsh, 1957), as a catalyst for the preparation of β-hexosamine penta-acetates (Stacey, 1944).

Finely powdered glucosamine hydrochloride (20 g., L. Light and Co.) was added during 30 min. with frequent shaking to a solution of anhydrous granular $ZnCl_2$ (16·6 g., British Drug Houses Ltd.) in acetic anhydride (106 ml.); it was essential that the temperature should be maintained at 45–55°. Dissolution was complete after about a further 15 min. at 50°, and the solution was then poured with stirring into water (140 ml.) at 0°; NaOH (64 g.) in water (200 ml.) was added over 30 min. at a temperature of

40–50°. Separation of β-glucosamine penta-acetate began before the end of the alkali addition, and was completed by cooling the mixture for 1 hr. at 0°. The crystalline product (10 g., yield 28%) was filtered, freed from acid with ice-cold water, and used without further purification, m.p. 185–186°, $[\alpha]_D^{22}$ +1·5° in $CHCl_3$ (*c*, 4). Hudson & Dale (1916) give m.p. 188–189°, $[\alpha]_D^{20}$ +1·2° for β-glucosamine penta-acetate.

A melt at 130° of the β-glucosamine penta-acetate (20·6 g.), *p*-nitrophenol (24·6 g.) and toluene-*p*-sulphonic acid (0·14 g.) was maintained at 120° for 2 hr. with occasional stirring, and $CHCl_3$ (800 ml.) was then added. The solution was washed thoroughly with water at 0°, then with 0·1 N-NaOH (total 600 ml.) at 0° until the aqueous phase suddenly changed in colour from yellow to dark brown. The $CHCl_3$ solution was rewashed with water at 0° and dried over $CaCl_2$; evaporation to small bulk yielded a product which was recrystallized from ethanol–$CHCl_3$ (1:1, 130 ml.) to give almost pure *p*-nitrophenyl β-glucosaminide tetra-acetate (8·8 g., yield 35%); m.p. 233°, $[\alpha]_D^{20}$ −40° in acetone (*c*, 0·5). Westphal & Schmidt (1952) give m.p. 241°, $[\alpha]_D^{18}$ −46·75° in pyridine for this compound.

De-*O*-acetylation of the tetra-acetate (13·2 g.) in solution in methanol–$CHCl_3$ (1:1, 240 ml.) was done with 0·1 N-sodium methoxide in methanol (7·3 ml.), added to the boiling solution under reflux. The mixture was immediately left to cool and kept at 0° overnight, and the product (8·9 g., 92% of the theoretical), m.p. 204°, was filtered off and washed with methanol at 0°. Pure anhydrous *p*-nitrophenyl *N*-acetyl-β-glucosaminide, m.p. 210°, $[\alpha]_D^{22}$ −18° in aqueous acetone (1:1) (*c*, 1), was obtained after two recrystallizations from boiling water (the compound decomposes slowly in aqueous solution above 50° to liberate *p*-nitrophenol). According to Westphal & Schmidt (1952) the monohydrate has m.p. 204°, $[\alpha]_D^{16}$ −25·5° in pyridine.

2-Acetamido-2-deoxygluconolactone. (*a*) By acetylation of glucosaminic acid. A modification of the method (Karrer & Mayer, 1937) of *N*-acetylation of chondrosaminic (galactosaminic) acid was used. Glucosaminic acid (3·1 g.), obtained by oxidation of glucosamine with freshly-precipitated yellow mercuric oxide (Hope & Kent, 1955), was dissolved in 10% (w/v) NaOH (26 ml.) and treated with acetic anhydride (8 ml.). The mixture rapidly warmed up, and after 15 min. was treated with dil. H_2SO_4 to give pH 2–3. Evaporation to dryness, followed by several evaporations with ethanol to remove the last traces of water, gave a resinous mass which was heated at 100° for 1 hr. *in vacuo*. This was then extracted twice with hot ethanol (total 300 ml.) and evaporated to dryness; the residue was re-extracted with hot ethanol (75 ml.), treated with charcoal and evaporated at minimum temperature to a clear gum. Extensive drying *in vacuo* yielded *2-acetamido-2-deoxygluconolactone* as a non-crystalline white powder (3·3 g., 95% of the theoretical) (Found: C, 42·8; H, 6·3; N, 5·8; $CH_3 \cdot CO$, 19·0. $C_8H_{13}O_6N$ requires C, 43·8; H, 6·0; N, 6·4; $CH_3 \cdot CO$, 19·6%).

This extremely deliquescent substance had initial $[\alpha]_D^{20}$ +81° in water (*c*, 2); the aqueous solution was acid (pH 3·5 at 25 mM) and reduced Fehling's solution slowly at 100°. Titration under N_2 with 0·01 N-NaOH to pH 7 gave an equivalent of 221 (theoretical value 219); it behaved almost entirely as a lactone with the ring opening readily at pH 6·4–6·5. The acetyl group was lost on treatment both with acid (see below) and with alkali; thus there was complete liberation of one equivalent of acetic acid in 0·5 N-NaOH at 100° after 1 hr.

(*b*) By oxidation of *N*-acetylglucosamine. Heyns & Koch (1953) found that glucosamine in the presence of a Pt catalyst was readily oxidized by gaseous O_2 to glucosaminic acid, but were unable to oxidize *N*-acetylglucosamine similarly. This was accomplished, however, by the procedure previously used to oxidize aliphatic hexosides to the conjugated hexuronic acids (Marsh, 1952), and also with freshly precipitated HgO at slightly alkaline pH.

(i) *N*-Acetylglucosamine (1 g., L. Light and Co.) in aqueous solution (25 ml.) with Pt catalyst (0·2 g.) was oxidized with gaseous O_2 at 40–45°, the mixture being kept neutral to litmus by frequent addition of 0·5 N-$NaHCO_3$ [see Marsh (1952) for details of catalyst preparation etc.]. When about one equivalent (9·0 ml.) of alkali had been added, the rate of change of pH was considerably decreased. At this point the solution was filtered and an insoluble lead salt was precipitated with basic lead acetate. Treatment with H_2S yielded a gum which was extracted with hot acetone, evaporated, and precipitated with ether from cold butanol solution, to yield a very deliquescent solid (0·14 g.).

(ii) To $HgCl_2$ (12·5 g.) in water (400 ml.) at room temperature was added 3 N-NaOH (33 ml.) with shaking; the precipitated yellow HgO was removed, washed with water by centrifuging, and resuspended in water (130 ml.). *N*-Acetylglucosamine (3·0 g.) in water (33 ml.) was added and after heating to 100°, 0·5 N-NaOH (30 ml., 1 equiv.) was added dropwise with mechanical stirring during 2 hr. to keep the pH at 7·0–8·5; immediate reduction of HgO was observed. Mercuric ion was removed by centrifuging followed by treatment with H_2S; the aqueous solution after treatment with charcoal was acidified (pH 2–3) with dil. H_2SO_4 and treated as in (*a*) to give a highly deliquescent product (1·8 g.).

Products obtained by oxidation of *N*-acetylglucosamine appeared to be similar to that obtained by *N*-acetylation of glucosaminic acid, but had slightly lower inhibitory power for β-*N*-acetylglucosaminidase. These alternative methods of preparation afforded evidence of the identity of the product, but were less satisfactory and not investigated further.

2-Acetamido-2-deoxygalactonolactone. The method of Karrer & Mayer (1937) was modified as follows. An aqueous solution (30 ml.) of galactosamine hydrochloride (1·22 g.) was added to a suspension of freshly prepared HgO (5 g.) in water (120 ml.). The mixture was heated for 20 min. on the steam bath; there was obvious formation of free Hg. After being centrifuged, the solution was treated with H_2S, filtered and concentrated to small bulk and an equal volume of ethanol added. The galactosaminic acid was filtered off and recrystallized from water with the addition of a little ethanol; yield 276 mg. (26%).

A solution of galactosaminic acid (314 mg.) in 10% (w/v) NaOH (2·7 ml.) was treated with acetic anhydride (0·5 ml.). After 20 min. the liquid was adjusted to about pH 2·5 with dil. H_2SO_4; the residue after evaporation to dryness was heated at 100° *in vacuo* for 45 min., then extracted twice with hot ethanol (total 20 ml). The filtered ethanolic solution was again evaporated to give a glassy solid (304 mg.), which after recrystallization from ethanol gave the pure 2-acetamido-2-deoxygalactonolactone (*N*-acetylgalactosaminonolactone), m.p. 168–169° (decomp.) with

sintering at 165°, $[\alpha]_D^{22} - 24°$ in water (*c*, 1). Yield 125 mg.; a second crop, m.p. 166–167° (decomp.) (28 mg.), was obtained from the mother liquors (total 39% of the theoretical). Karrer & Mayer (1937) give m.p. 165° for this compound (Found: C, 44·1; H, 5·9; N, 6·1. Calc. for $C_8H_{13}O_6N$: C, 43·8; H, 6·0; N, 6·4%).

An aqueous 20 mM solution of *N*-acetylgalactosaminonolactone had pH 6·1; it behaved entirely as a lactone with ring fission at pH 7·5. Titration with 0·01 N-NaOH under N_2 to pH 8·5, followed by back titration to pH 7 with standard acid, gave an equivalent weight of 227 (theoretical for $C_8H_{13}O_6N$, 219). The lactone in aqueous solution slowly reduced Fehling's solution at 100°.

Unfortunately, lack of material was a limiting factor in the experiments with the galactonolactone. In particular, it precluded measurements of changes in rotation during alterations in inhibitory power.

Buffer solutions

Buffer solutions were prepared by titrating the calculated weight of the free acid (citric acid, glycine, H_3PO_4) to the required pH with NaOH solution.

Enzyme preparations

From rat epididymis. The combined caput and cauda epididymis (total about 1 g.) of an adult rat was homogenized in water (8 ml.). According to Pugh, Leaback & Walker (1957*a*) complete solubilization of tissue β-*N*-acetylglucosaminidase requires the presence of NaCl; 1 ml. of 0·1 M-NaCl and 1 ml. of 0·5 M-citric acid–NaOH buffer, pH 4·3, was added and the mixture incubated at 38° for 1 hr. and centrifuged at 1500 *g* for 15 min. The supernatant was fractionated with ammonium sulphate and the precipitate obtained between 20 and 80% saturation with $(NH_4)_2SO_4$ was redissolved in water (10 ml./g. of tissue for assay of β-*N*-acetylglucosaminidase and 2 ml./g. of tissue for α-*N*-acetylglucosaminidase activity). The appropriate stock solution was further diluted 1:30 for assay with phenyl *N*-acetyl-β-glucosaminide, and 1:100 for assay with *p*-nitrophenyl *N*-acetyl-β-glucosaminide.

From limpet. The procedure was as described by Levvy, Hay & Marsh (1957). Before assay with the different substrates, the stock solution (2·5 ml./g. of tissue) was diluted 1:10 for phenyl *N*-acetyl-β-glucosaminide, 1:60 for *p*-nitrophenyl *N*-acetyl-β-glucosaminide and 1:3 for phenyl *N*-acetyl-α-glucosaminide.

Enzyme assay

Preliminary experiments often showed a slight variable activation (not exceeding 10%) of epididymal β-*N*-acetylglucosaminidase activity in the presence of NaCl (cf. Pugh *et al.* 1957*a*). In general, therefore, all buffers contained NaCl to give a final concentration of 0·1 M in the enzyme assay.

Liberation of phenol. The hydrolysis of phenyl *N*-acetyl-α- and -β-glucosaminides was followed by the method of Kerr, Graham & Levvy (1948), free phenol being measured on the Spekker absorptiometer with Ilford no. 608 red filters (peak transmission 680 mμ). The incubation mixture (1 ml.) contained 0·1 ml. of 0·5 M citric acid–NaOH buffer, 0·5 ml. of substrate (0·02 M) and 0·2 ml. of enzyme solution. The buffer pH was adjusted to give a final value of 4·3 in the assay; this was shown by Pugh *et al.* (1957*b*) to be the pH optimum for rat-kidney β-*N*-acetylglucosaminidase, and was accepted arbitrarily for assay of rat-epididymis and limpet α- and β-*N*-acetylglucosaminidases.

In addition to the usual controls, it was necessary to correct for interference at concentrations greater than 0·1 mM of *N*-acetylglucosaminonolactone and *N*-acetylgalactosaminonolactone, owing to their reducing action.

Liberation of p-*nitrophenol.* The mixture incubated (4 ml.) contained 1 ml. of 0·2 M-citric acid–NaOH buffer, 2 ml. of 10 mM-*p*-nitrophenyl *N*-acetyl-β-glucosaminide, and 0·5 ml. of enzyme solution, the final pH being 4·4 (Heyworth *et al.* 1957). After incubation for 1 hr. at 38°, 4 ml. of 0·4 M glycine–NaOH buffer, pH 10·5, was added, the solution centrifuged, and the liberated *p*-nitrophenol in the supernatant measured on the Spekker absorptiometer with Ilford no. 601 violet filters (peak transmission 430 mμ).

For studies of the changes in inhibitory power of the lactones and their derivatives, only rat-epididymis enzyme and 5 mM-*p*-nitrophenyl *N*-acetyl-β-glucosaminide were used. Whereas the β-*N*-acetylglucosaminidase activity of stock epididymis preparations kept at 0° was constant over several weeks, when diluted for assay its activity decreased at a rate of about 5%/hr. at 0° (cf. Pugh *et al.* 1957*b*).

Experiments on changes in the inhibitory power of the lactones under varying conditions of pH were so arranged that dilution of the inhibitor solution before assay was great enough to preclude any effect on the pH of the incubation mixture in the enzyme assay.

RESULTS

Comparison of α- and β-N-acetylglucosaminidase activities of rat epididymis and limpet

The α- and β-*N*-acetylglucosaminidase activities of typical rat-epididymis and limpet-visceral-hump preparations are shown in Table 1. The ratios of β to α enzymic activities, with 10 mM-phenyl glycosides as substrates, were 615 and 4 for the epididymis and visceral preparations respectively. Rat epididymis was a much better source of β-*N*-acetylglucosaminidase activity, but the limpet was the more convenient source of the α-enzyme. Both preparations hydrolysed *p*-nitrophenyl *N*-acetyl-β-glucosaminide more readily than the phenyl β-glycoside, but whereas the ratio of μg. of *p*-nitrophenol to μg. of phenol liberated by epididymis was 2·4, identical with that for ram-testis preparations (Dr P. G. Walker, personal communication), the corresponding value for limpet was 10. We were unable to confirm the relatively high α-*N*-acetylglucosaminidase activity reported by Roseman & Dorfman (1951) for certain other rat tissues such as liver and testis. Epididymis displayed the highest activity observed for this enzyme in the rat.

The liberation of *p*-nitrophenol from the β-glycoside was found to be proportional to the amount of enzyme present and to the period of incubation for at least 1 hr., for both epididymis (cf. Fig. 2 below) and limpet preparations.

Table 1. *α- and β-N-Acetylglucosaminidase activities of rat-epididymis and limpet-visceral-hump preparations*

The figures represent μg. of aglycone liberated/hr./g. of tissue at 38° from 10 mM-phenyl *N*-acetyl-α- and -β-glucosaminides at pH 4·3, and from 5 mM-*p*-nitrophenyl *N*-acetyl-β-glucosaminide at pH 4·4, in 0·05 M citric acid–NaOH buffer.

Substrate	Enzyme activity (μg./g. of tissue) Epididymis	Limpet
Phenyl *N*-acetyl-α-glucosaminide	102	1 200
Phenyl *N*-acetyl-β-glucosaminide	62 800	4 600
p-Nitrophenyl *N*-acetyl-β-glucosaminide	151 000	47 000

Table 2. *Inhibition by freshly prepared solutions of N-acetylglucosaminonolactone and N-acetylgalactosaminonolactone of the α- and β-N-acetylglucosaminidase activities in rat epididymis and limpet*

The substrates were 10 mM-phenyl *N*-acetyl-α- and -β-glucosaminides.

Inhibitor	Concn. (μM)	Inhibition (%) Epididymis α-Enzyme	Epididymis β-Enzyme	Limpet α-Enzyme	Limpet β-Enzyme
N-Acetylglucosaminonolactone	1000	47	100	22	96
N-Acetylglucosaminonolactone	2	0	67	0	0
N-Acetylgalactosaminonolactone	1000	0	98	0	57
N-Acetylgalactosaminonolactone	10	—	54	—	0

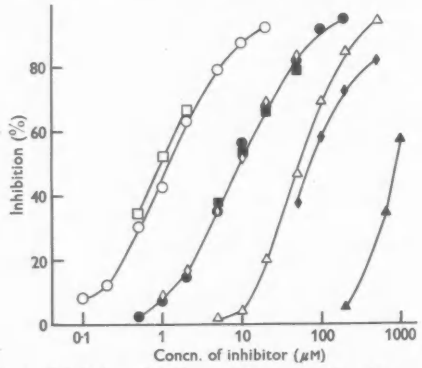


Fig. 1. Inhibition of β-*N*-acetylglucosaminidase by varying concentration of lactone inhibitors. Inhibitor, *N*-acetylglucosaminonolactone: substrate, 10 mM-phenyl *N*-acetyl-β-glucosaminide, enzyme from rat epididymis (□) and limpet (△); substrate 5 mM-*p*-nitrophenyl *N*-acetyl-β-glucosaminide, enzyme from rat epididymis (○) and limpet (◇). Inhibitor, *N*-acetylgalactosaminonolactone: substrate 10 mM-phenyl *N*-acetyl-β-glucosaminide, enzyme from rat epididymis (■) and limpet (▲); substrate, 5 mM-*p*-nitrophenyl *N*-acetyl-β-glucosaminide, enzyme from rat epididymis (●) and limpet (◆). Inhibitor concentrations are plotted on a logarithmic scale.

Prolongation of incubation beyond this time often caused slight diminution of the rate of hydrolysis, presumably due to enzyme instability, even in the presence of substrate. These observations are in general agreement with those of Pugh *et al.* (1957*b*).

Inhibition of epididymis and limpet α- and β-N-acetylglucosaminidase by the lactones

With the phenyl glycosides as substrates, it was shown that both *N*-acetylglucosaminonolactone and *N*-acetylgalactosaminonolactone were very powerful inhibitors of epididymal β-*N*-acetylglucosaminidase and less effective towards the same enzyme from the limpet, whereas only the former lactone, and then at relatively high concentration, inhibited α-*N*-acetylglucosaminidase from either source (Table 2).

Further evidence for the non-identity of α- and β-*N*-acetylglucosaminidase was obtained in an experiment in which 5 mM-phenyl *N*-acetyl-α-glucosaminide had no effect on the hydrolysis of 2·5 mM-*p*-nitrophenyl *N*-acetyl-β-glucosaminide by the epididymal enzyme, whereas 5 mM-phenyl *N*-acetyl-β-glucosaminide caused 40% depression of nitrophenol liberation.

Inhibition of the β-glycosidase from both sources was studied at varying concentrations of both lactones (freshly prepared solutions), with 10 mM-phenyl and 5 mM-*p*-nitrophenyl *N*-acetyl-β-glucosaminide as substrates. The results are shown in Fig. 1 and the derived values for the lactone concentrations giving 50% inhibition are given in Table 3.

Both lactones always acted as competitive inhibitors of β-glucosaminidase, and K_m and K_i were calculated by the method of Lineweaver & Burk (1934); mean values are given in Table 3. In the case of the epididymal enzyme, K_i for either lactone was very similar with both substrates

employed, and *N*-acetylglucosaminonolactone had approximately five times the affinity ($1/K_i$) of *N*-acetylgalactosaminonolactone. Values of K_m are of the same order as those given by Pugh *et al.* (1957*b*) for a rat-kidney preparation and by Heyworth *et al.* (1957) for the enzyme in ram testis.

Values of K_m for the limpet enzyme were about ten times those for the epididymal enzyme. Both lactones were more powerful inhibitors for the hydrolysis of *p*-nitrophenyl than of phenyl *N*-acetyl-β-glucosaminide by the limpet enzyme, and this is reflected in the differing K_i values for *N*-acetylglucosaminonolactone, again the more powerful of the two lactones. This variation in K_i with differing substrates was previously encountered in studying the effects of saccharo-1:4-lactone and galactosaccharolactone on the hydrolysis of phenyl and *o*-nitrophenyl β-galacturonide by preparations from the limpet and the female rat preputial gland (Levvy, McAllan & Marsh, 1958). It was then considered more probable that the nitro group modified the mode of combination of substrate with enzyme, rather than that the effect was due to the presence of two enzymes of different aglycone specificity.

The results shown in Fig. 1 for the inhibition by *N*-acetylgalactosaminonolactone of the epididymal enzyme acting on both phenyl and *p*-nitrophenyl *N*-acetyl-β-glucosaminide, and for the inhibition by *N*-acetylglucosaminonolactone of the limpet enzyme acting on phenyl *N*-acetyl-β-glucosaminide fall on the same curve. This is an accidental phenomenon resulting from similarities in the ratio of K_i to K_m, together with the selection of the substrate concentration employed in studying the effect of varying inhibitor concentration.

Nature of the inhibitory principle in solutions of the aldonolactones

General comments. Before describing the diverse inhibitory effects of the lactones under different conditions of pH, it might be useful to mention the types of structural change which these compounds could feasibly undergo, namely (*a*) hydrolysis of the lactone ring to give the free acid or anion, (*b*) interconversion of one lactone form into another, (*c*) removal of the *N*-acetyl group and (*d*) any other change in structure. Conchie (1954) studied the changes of inhibitory power of glucono-1:4- and -1:5-lactones for β-glucosidase due to mutarotation in solution; in this case both lactones caused similar inhibition of the enzyme, but from considerations of the very specific requirements of lactone structure for glycosidase inhibition (Conchie & Levvy, 1957) this would not necessarily appear to be a general rule. The following hypotheses are therefore proposed in an attempt to reconcile the complex changes observed to occur in the experiments outlined below.

From its method of preparation and rotation the solid *N*-acetylglucosaminonolactone was probably an equilibrium mixture of the 1:4- and 1:5-lactones, both of which caused similar inhibition of β-*N*-acetylglucosaminidase. There was preferential formation of one species from the free acid in solution at low pH, followed by attainment of the equilibrium. Both lactones in the equilibrium mixture were very susceptible to alkaline hydrolysis and could not be distinguished by titration, nor by differential loss of acetyl in strongly acid solution. In addition to these changes, *N*-acetylglucosaminonolactone in solution showed a slow irreversible decomposition, largely independent of pH; Bergmann, Zervas & Silberkweit (1931) found that the product of partial acetylation of glucosaminic acid decomposed spontaneously to give an α-pyrone. The crystalline, sharply melting *N*-acetylgalactosaminonolactone, however, was almost certainly a single species, probably the 1:4-lactone from its relative stability to alkaline hydrolysis. In solution, particularly at about pH 5–6, there was formation of an equilibrium mixture containing the 1:5-lactone, which had a considerably greater

Table 3. *Dissociation constants for the complexes formed by rat epididymis and limpet β-N-acetylglucosaminidases with substrate (K_m) and lactone inhibitors (K_i), and the inhibitor concentration required for 50% inhibition of hydrolysis*

The substrates were phenyl and *p*-nitrophenyl *N*-acetyl-β-glucosaminides (for details of assay, see Table 1). The amount of lactone required for 50% inhibition was read from Fig. 1.

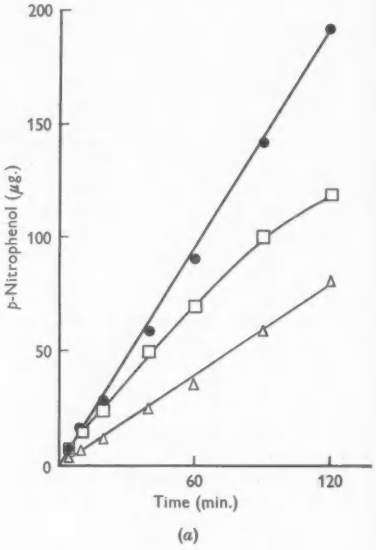
			Inhibitor			
			N-Acetylglucosaminonolactone		*N*-Acetylgalactosaminonolactone	
Enzyme	Substrate aglycone	K_m (mM)	K_i (μM)	Concn. for 50% inhibition (μM)	K_i (μM)	Concn. for 50% inhibition (μM)
Epididymis	Phenol	0·71	0·072	1·0	0·40	8·3
Epididymis	*p*-Nitrophenol	0·40	0·090	1·3	0·41	8·3
Limpet	Phenol	6·4	27	54	—	850
Limpet	*p*-Nitrophenol	3·8	4·0	8·3	32	75

inhibitory effect upon β-*N*-acetylglucosaminidase, but which was much more unstable at neutral pH. *N*-Acetylgalactosaminic acid at low pH underwent preferential formation of the 1:5-lactone, followed by establishment of an equilibrium mixture, the composition of which depended upon the pH of the solution.

Inhibition by N-*acetylglycosaminate.* Solutions of *N*-acetylglucosaminonolactone and *N*-acetylgalactosaminonolactone showed no perceptible change in inhibitory power during 2 hr. incubation with enzyme and substrate at pH 4·4 and 38° (Fig. 2). In Fig. 2 (*a*), a linear curve was obtained in presence of *N*-acetylglucosaminonolactone, as in the control. In the experiment shown in Fig. 2 (*b*), the enzyme displayed significant inactivation in the control when incubation was prolonged beyond 1 hr., and this was reflected in the curve for inhibition by *N*-acetylgalactosaminonolactone, which was, however, linear over the first 90 min. After incubation for 1 hr. in the usual way, the *N*-acetylglycosaminic acids obtained by opening the lactone ring with alkali were found to give only feeble inhibition (5–10%) of β-*N*-acetylglucosaminidase at concentrations of 2 μM for the glucosamine derivative and 10 μM for the galactosamine derivative at which the untreated lactones caused 60% inhibition. This small residual effect was due, not to inherent inhibition by the *N*-acetylglycosaminate ion, but to partial closing of the lactone ring under the acid conditions of enzyme assay, as can be seen from Fig. 2, in which the anion concentration is 20 μM for the glucosamine derivative and 100 μM for that of galactosamine. The rate of enzymic hydrolysis, initially unchanged in the presence of the anion, progressively decreased during the period of incubation. Development of inhibitory power was more clearly seen with *N*-acetylgalactosaminate than with *N*-acetylglucosaminate (cf. also Fig. 6).

pH *stability of the lactones.* Incubation of the lactones at 37° and varying pH before assay in the usual way gave the results shown in Fig. 3. Neither inhibitor showed any change after 1 hr. at pH 3. After incubation for 1 hr. at a pH greater than 4, *N*-acetylglucosaminonolactone showed an increasing loss of inhibitory power with pH; after 1 hr. at pH 8, there was only a small residual inhibitory action, due no doubt to relactonization during the assay. Pretreatment of *N*-acetylglucosaminonolactone for 24 hr. caused an appreciable loss in activity even at pH 3.

In contrast, the maximum inhibitory power of *N*-acetylgalactosaminonolactone was displayed only after pre-incubation for 1 hr. at pH 5–6, indicating that a new and more inhibitory compound was formed. Inhibition was not completely abolished after 1 hr. at pH 9. Minimum inhibition,



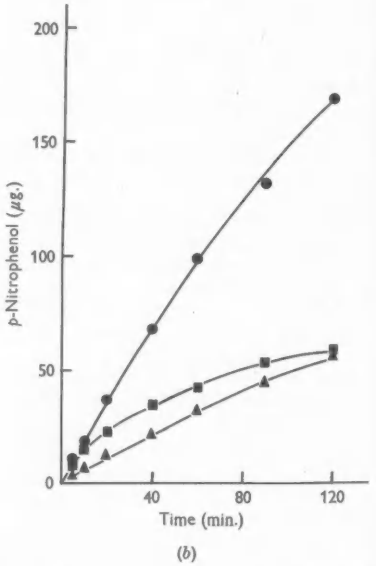


Fig. 2. Hydrolysis of 5 mM-*p*-nitrophenyl *N*-acetyl-β-glucosaminide in the absence (●) and presence of (*a*) 2 μM-*N*-acetylglucosaminonolactone (△) and 20 μM-*N*-acetylglucosaminate (□), (*b*) 10 μM-*N*-acetylgalactosaminonolactone (▲) and 100 μM-*N*-acetylgalactosaminate (■).

corresponding to the figure for the anion, was reached after 24 hr. incubation at pH 7·5.

Solutions of *N*-acetylglucosaminonolactone in water (pH 3·2) slowly lost activity on standing at 0° (Fig. 4). Identical results were obtained in HCl (pH 2·0) at 37°, suggesting that neither loss of acetyl nor opening of the lactone ring was involved. In fact, it was not found possible to keep this inhibitor in aqueous solution without at least this fall in inhibitory power (about 12 % in 24 hr.). If the solid was exposed to moist air, deliquescence was accompanied by a similar and irreversible loss of inhibitory power.

Aqueous solutions of *N*-acetylgalactosaminonolactone (pH 6) kept at 0° increased in inhibitory power during the first 24 hr. (Fig. 4, cf. Fig. 3), by which time a stable equilibrium was apparently reached, with little further change of inhibitory power over an interval of 6 days.

The greater retention of inhibitory power by *N*-acetylgalactosaminonolactone at physiological pH is shown also in Fig. 5. After incubation of this lactone in H_3PO_4–NaOH buffer, pH 7, at 38°, there was an initial rise in inhibitory power, followed by a fall, presumably due to opening of the lactone ring, but even after 3 hr. incubation the residual activity was only a little less than that of the starting material. In contrast with this, a solution of *N*-acetylglucosaminonolactone at pH 7 fell to its minimum inhibitory activity very rapidly.

Loss of inhibitory power due to loss of the acetyl group at acid pH is also shown in Fig. 5. After heating in 0·5N-HCl at 100° for 2 hr., *N*-acetylglucosaminonolactone solutions were non-inhibitory. Acetyl determinations showed that one equivalent of acetic acid was liberated by this treatment, and glucosaminic acid was isolated as follows. Treatment of *N*-acetylglucosaminonolactone (100 mg.) in 0·5N-HCl (5 ml.) at 100° for 2 hr., followed by adjustment of pH to 4·1 with NaOH, concentration to approximately 1 ml. and addition of an equal volume of methanol, yielded glucosaminic acid (58 mg., 65 % of the theoretical). After recrystallization from water with addition of methanol, this had $[\alpha]_D^{20} - 6°$ in water (*c*, 2) and $[\alpha]_D^{20} - 15°$ in 0·5N-HCl (*c*, 2); Hope & Kent (1955) give $[\alpha]_D^{23}$ 0 ± 2° in water (*c*, 0·83), $[\alpha]_D^{21} - 16·5°$ in 2·5 % HCl (*c*, 1·64). 10 mM-Glucosaminic acid, before or after heating at pH 2 for 1 hr. at 100° to effect any possible lactonization, had no effect on β-*N*-acetylglucosaminidase activity.

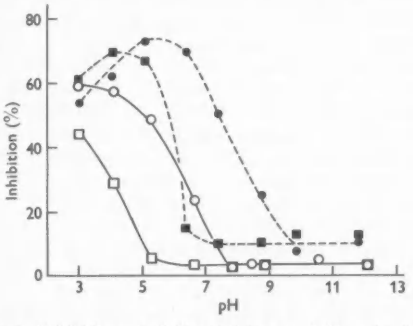


Fig. 3. Inhibition of β-*N*-acetylglucosaminidase by lactones pre-incubated in citrate buffer at 38° at varying pH; 2 μM-*N*-acetylglucosaminonolactone incubated for 1 hr. (○) and 24 hr. (□); 10 μM-*N*-acetylgalactosaminonolactone incubated for 1 hr. (●) and 24 hr. (■).

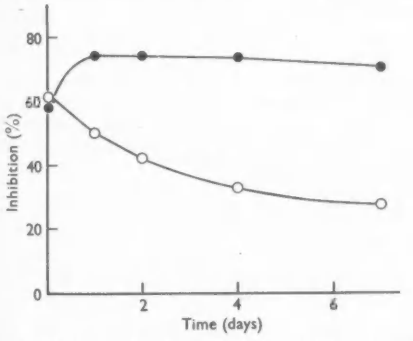


Fig. 4. Inhibition of β-*N*-acetylglucosaminidase by 2 μM-*N*-acetylglucosaminonolactone (○) and by 10 μM-*N*-acetylgalactosaminonolactone (●) kept at 0° in aqueous mM solution for varying periods.

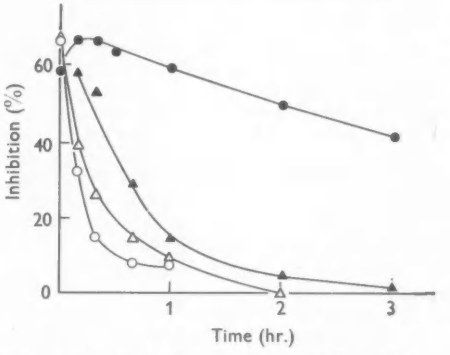


Fig. 5. Inhibition of β-*N*-acetylglucosaminidase by 2 μM-*N*-acetylglucosaminonolactone incubated in phosphate buffer, pH 7, at 38° (○) and in 0·5N-HCl at 100° (△), and by 10 μM-*N*-acetylgalactosaminonolactone incubated in phosphate buffer, pH 7, at 38° (●) and in 2N-HCl at 100° (▲).

The acetyl group of *N*-acetylgalactosaminonolactone was more stable to acid hydrolysis (Fig. 5). Heating in 2N-HCl at 100° caused almost complete loss of inhibitory power in 3 hr.; in 0·5N-HCl at 100° the loss was slow.

Formation of lactone in N-acetylglycosaminate solutions. As previously described (see Fig. 2) the inhibitory power of the lactones, completely lost by conversion into the sodium salts of the free acids, was slowly restored at the slightly acid pH of enzyme assay. After 2 hr. incubation at 38° and pH 2 solutions of *N*-acetylglucosaminate attained their original inhibitory power, and at 100° this value was reached in 20 min. at pH 3 (Fig. 6*a*). This maximum was followed by a steady loss of inhibitory power at pH 3 and 100°, probably due to loss of the acetyl group. Sodium *N*-acetylglucosaminate, calculated as the lactone, had $[\alpha]_D^{20}+1°$ in water (*c*, 2); when maximum inhibitory power (61% inhibition at 2 μM) had been restored after 2 hr. at 38° and pH 2, the optical rotation was $[\alpha]_D^{20}+37°$. The solution was then evaporated and the gum dried over anhydrous $CaCl_2$ *in vacuo* for 48 hr., whereupon the optical rotation had increased to $[\alpha]_D^{20}+77°$, although the inhibitory power was unchanged. There was thus a species change that was without effect upon the inhibitory power, and the final product was apparently identical with the original *N*-acetylglucosaminonolactone ($[\alpha]_D+81°$).

Bringing *N*-acetylgalactosaminate solutions to pH 2 or 3, however, produced very different effects (Fig. 6*b*). Maximum inhibitory activity was attained after the same period as with *N*-acetylglucosaminonolactone, but the inhibitory power was then much greater than anything yet displayed by the original lactone, and higher even than that of *N*-acetylglucosaminonolactone. At pH 3 and 100° subsequent incubation again caused loss of inhibitory power. At pH 4 and 100° a lower activity (comparable with that seen in Fig. 3) was attained after 30 min. incubation and was unaltered by further incubation.

It was obviously desirable to study the properties of the very inhibitory entity initially formed in acidified solutions of *N*-acetylgalactosaminate; solutions heated for 20 min. at 100° and pH 3 were used. The resulting solution, at a concentration corresponding to 0·65 μM of the original *N*-acetylgalactosaminonolactone, caused 50% inhibition of hydrolysis of 5 mM-*p*-nitrophenyl *N*-acetyl-β-glucosaminide by epididymis enzyme (cf. the figure of 8·3 μM given in Table 3). The inhibitory principle was quite stable for at least 2 hr. at 38° and pH 4·4, as shown by its effect in enzyme assays, but was very unstable at pH 7 in H_3PO_4–NaOH buffer (Fig. 7), as one would expect for the 1:5-lactone. Even addition of the buffer before immediate transfer to the assay caused appreciable

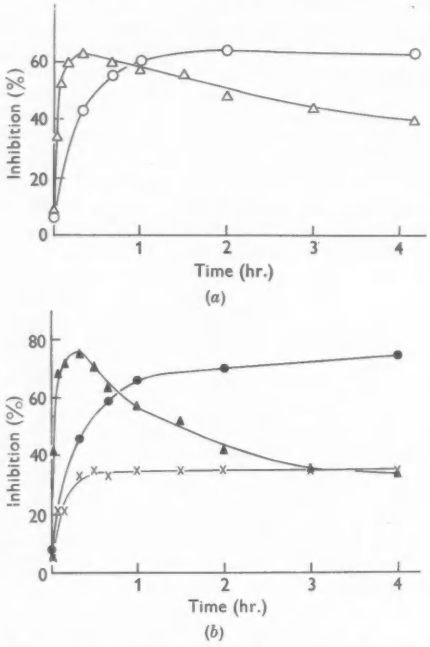


Fig. 6. Inhibition of β-*N*-acetylglucosaminidase by (*a*) 2 μM-*N*-acetylglucosaminate incubated at pH 2 and 38° (○) and at pH 3 and 100° (△); (*b*) 2 μM-*N*-acetylgalactosaminate incubated at pH 2 and 38° (●), pH 3 and 100° (▲) and at pH 4 (10 mM-citrate buffer) and 100° (×). The liquids were adjusted to pH 2 and 3 with HCl.

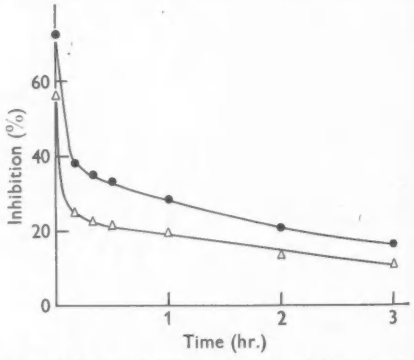


Fig. 7. Inhibition of β-*N*-acetylglucosaminidase by 5 μM-(●) and 2 μM- (△) -*N*-acetylgalactosaminate after 20 min. at pH 3 and 100°, followed by varying periods in phosphate buffer, pH 7, at 38°.

loss of inhibitory power. On incubation of the buffered solution at 38°, the inhibitory power fell rapidly to the normal values for *N*-acetylgalactosaminonolactone at this concentration, namely 38% inhibition at 5 μM and 25% inhibition at 2 μM (see Fig. 1). Thereafter there was a slow loss of inhibitory power, at the rate shown by the crystalline *N*-acetylgalactosaminonolactone at 38° and pH 7 (Fig. 5).

Effects of other N*-substituted glucosaminonolactones*

N-Benzoyl- and *N*-toluene-*p*-sulphonyl-glucosaminonolactone (Hope & Kent, 1955) at mM concentration had no effect on the hydrolysis of 5 mM-*p*-nitrophenyl *N*-acetyl-β-glucosaminide by the epididymis enzyme. However, Neuberger & Pitt-Rivers (1939) observed similar hydrolysis of methyl *N*-formyl- and *N*-acetyl-β-glucosaminides by β-*N*-acetylglucosaminidase preparations from *Helix pomatia*, although other *N*-acyl β-glucosaminides were unattacked.

DISCUSSION

This work provides further evidence on the one hand for the general nature of aldonolactone inhibition of glycosidases acting on substrates of the same configuration, and on the other hand for the value of such enzymic studies in the chemistry of sugar acid lactones. It has enabled us to isolate the hitherto unknown 2-acetamido-2-deoxygluconolactone, and has provided evidence for the existence in solution of a second 2-acetamido-2-deoxygalactonolactone, probably the 1:5-lactone. The isolation of this compound seems not impossible, since the slightly impure second crop obtained in the preparation of the 1:4-lactone was somewhat more inhibitory in freshly prepared solution than the main crop.

Since there was little to choose between 2-acetamido-2-deoxygluconolactone and the galactonolactone as inhibitors for β-*N*-acetylglucosaminidase, it would appear that, in agreement with Heyworth *et al.* (1957), a single enzyme is responsible for the hydrolysis of both *N*-acetyl-β-glucosaminides and -galactosaminides. The lactones, however, clearly distinguish between α- and β-*N*-acetylglucosaminidase, in agreement with the suggestion of Roseman & Dorfman (1951) that these are different enzymes. An analogous situation has already been described for enzymes hydrolysing glucuronides and galacturonides (Marsh & Levvy, 1958). In the same way α- and β-galactosidase could be distinguished by the action of galactonolactone, although gluconoand mannono-lactone inhibited their respective α- and β-glycosidases equally powerfully (Conchie & Levvy, 1957).

The *N*-acetylglycosaminonolactones are at least as powerful enzyme inhibitors as saccharo-1:4-lactone, the inhibitor for β-glucuronidase. The latter, as already noted by Conchie & Levvy (1957), is much more efficient than the simpler aldonolactones, such as gluconolactone, and it would seem that the presence of a second 'functional' group in the inhibitor molecule enhances its action.

Conchie & Levvy (1957) showed that β-*N*-acetylglucosaminidase showed no appreciable inhibition by any of the aldonolactones that they studied, and such other glycosidases as we have examined (β-glucuronidase, β-galactosidase, α-mannosidase) are not appreciably affected by mM-2-acetamido-2-deoxygluconolactone, proving that inhibition is highly specific for both enzyme and lactone.

The relative abundance of α-*N*-acetylglucosaminidase activity in the limpet is of particular interest since Neuberger & Pitt-Rivers (1939) could not detect this enzyme in *Helix pomatia*, although Utusi, Huzi, Matumoto & Nagaoka (1949) have reported trace activity in β-*N*-acetylglucosaminidase preparations from other snails. Herein lies yet another analogy between the glucuronidases and the *N*-acetylglucosaminidases, for the very high β-glucuronidase activity of the limpet is accompanied by an appreciable, but distinct, α-glucuronidase activity (Marsh & Levvy, 1958), whereas the snail possesses only the former enzyme (Utusi *et al.* 1949).

SUMMARY

1. 2-Acetamido-2-deoxygluconolactone (*N*-acetylglucosaminonolactone) was synthesized; this compound and 2-acetamido-2-deoxygalactonolactone (*N*-acetylgalactosaminonolactone) were very powerful competitive inhibitors of β-*N*-acetylglucosaminidase from rat epididymis.

2. These lactones also inhibited the β-*N*-acetylglucosaminidase in the limpet, but less powerfully, and only *N*-acetylglucosaminonolactone, and then only at high concentration, had any effect upon α-*N*-acetylglucosaminidase.

3. The free acids obtained by alkali treatment of the lactones were not inhibitors of β-*N*-acetylglucosaminidase, but some reconversion into the lactones occurred at the acid pH of the enzyme assay.

4. *N*-Acetylgalactosaminonolactone was more stable than *N*-acetylglucosaminonolactone in pure aqueous solution or in buffers at physiological pH.

5. The lactones and the acids derived from them underwent conversion in aqueous solution into equilibrium mixtures containing more than one species, the composition depending on the pH. In particular, there was some conversion of the known *N*-acetylgalactosaminonolactone into a more powerful but less stable inhibitor, probably the 1:5-lactone.

We are greatly indebted to Dr J. W. Minnis for C, H and N analyses, to Dr P. G. Walker for a gift of D-galactosamine hydrochloride and to Dr P. W. Kent for specimens of *N*-benzoyl- and *N*-toluene-*p*-sulphonyl-glucosaminonolactones.

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Crystalline *p*-Aminobenzylpenicillin: Preparation and some Properties

BY A. L. TOSONI, D. G. GLASS AND L. GOLDSMITH*
Connaught Medical Research Laboratories, University of Toronto

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Attempts to prepare *p*-aminobenzylpenicillin were made shortly after the role of precursors in penicillin fermentations became known. These attempts apparently failed (see Behrens, 1949, for review). The formation of *p*-aminobenzylpenicillin was reported by Corse, Jones, Soper, Whitehead & Behrens (1948), but no material was isolated. Brewer & Johnson (1953) described the preparation of a partially purified *p*-aminobenzylpenicillin by using *p*-aminophenylacetic acid as precursor. The levels of penicillin obtained in the fermentation were not given, the units/mg. of the material obtained were not indicated and it was stated that attempts at further purification had failed. It was reported, however, that the rate of decrease in concentration of *p*-aminobenzylpenicillin in the blood of dogs was slower than that of benzylpenicillin (penicillin G), that the rate of renal excretion was only one-third as fast as that for benzylpenicillin and that the material was somewhat more stable to acid than benzylpenicillin.

Owing to the presence of its aromatic amino group, *p*-aminobenzylpenicillin would be amenable to treatments yielding several types of derivatives. This fact, together with the properties indicated by Brewer & Johnson (1953), initiated, in our Laboratories, a search for a method of preparing pure *p*-aminobenzylpenicillin in sufficient quantities for a systematic study.

The work of Brewer & Johnson (1953), using *p*-aminophenylacetic acid as precursor, was repeated. Penicillin titres of about 1000 units/ml. were obtained, and the active material when isolated was a yellow-to-brown amorphous powder which assayed at about 300 units/mg. Attempts to further purify the material were not successful.

p-Aminobenzylpenicillin is more hydrophilic than *p*-hydroxybenzylpenicillin (penicillin X) and hence cannot be separated from impurities found in the fermentation liquors by the conventional methods of solvent extraction commonly used with benzylpenicillin. Similar difficulties have been encountered in the purification of another hydrophilic penicillin, aminocarboxybutylpenicillin (cephalosporin N, or synnematin B; Newton & Abraham, 1954).

Since *p*-aminobenzylpenicillin is intractable to purification by solvent extraction of fermentation

* Present address: Fermentation Products, Newaygo, Michigan, U.S.A.

liquors, it was thought that it might more readily be obtained in the pure state by chemical conversion from a readily purifiable non-hydrophilic biosynthetic penicillin. Accordingly, it was found that the non-hydrophilic *p*-nitrobenzylpenicillin was readily produced in the crystalline state and that on hydrogenation it gave a good yield of *p*-aminobenzylpenicillin.

RESULTS

p-*Nitrobenzylpenicillin*

p-Nitrobenzylpenicillin has been described by Behrens *et al.* (1948), but the amount obtained was not sufficient for recrystallization, and their methods of purification involved tedious chromatographic techniques. Their material assayed about 1700 units/mg.

In our work penicillin titres as high as 3400 units/ml. were obtained and the crystalline *p*-nitrobenzylpenicillin was isolated as the potassium salt by the usual techniques of purification. The material was identified by comparing its ultraviolet-absorption spectrum with that of *p*-nitrophenylacetic acid and by identification of the *p*-aminobenzylpenicillin produced by hydrogenation.

p-*Aminobenzylpenicillin*

Hydrogenation of *p*-nitrobenzylpenicillin at atmospheric or slightly higher pressures gave *p*-aminobenzylpenicillin. Among the various combinations of catalyst and solvent that were tried, palladium on charcoal as the catalyst and propan-2-ol as the solvent gave the best results. Evaporation *in vacuo* of the solution obtained on hydrogenation yielded crystalline *p*-aminobenzylpenicillin.

Some properties of p-*aminobenzylpenicillin*

Chemical and physical properties. The potassium salt of *p*-aminobenzylpenicillin, with an activity of 1100 units/mg., was a white crystalline powder, readily soluble in water but sparingly soluble in organic solvents. The product withstood a temperature of 110° for several hours without loss of potency. On chromatographic analysis 100% of the activity was found in the zone where penicillin X normally appears. The crystalline potassium salt was readily converted into the crystalline sodium salt by 'salting out' from a 20% (w/v) aqueous solution with sodium sulphate. It was not precipitated from concentrated aqueous solution by procaine, which forms a sparingly soluble salt with benzylpenicillin. However, like benzylpenicillin (Szabo, Edwards & Bruce, 1951), it was precipitated from aqueous solution by *NN*-dibenzylethylenediamine to give a crystalline product which was very sparingly soluble in water (about 1 mg./ml.).

It was more stable in aqueous acid solution (pH 2·3, 24°) than benzylpenicillin. Thus 29% of its activity remained after 167 min., whereas only 4% of the activity of benzylpenicillin was left after this time. *p*-Aminobenzylpenicillin is destroyed by preparations of penicillinase at a slightly lower rate than is benzylpenicillin (M. Goldner, personal communication, 1957).

Elementary analysis of the potassium salt gave the following result: C, 49·6; H, 4·5; N, 10·1; S, 8·2. $C_{16}H_{18}O_4N_2SK$ requires C, 49·6; H, 4·7; N, 10·8; S, 8·3%; $[\alpha]_D^{25} = +267°$ in water (7 mg./ml.). This material showed ultraviolet-absorption maxima at 238 mμ ($\log \epsilon = 4{\cdot}02$) and 285 m$\mu$ ($\log \epsilon = 3{\cdot}16$). The maxima for *p*-aminophenylacetic acid are 236 and 285 mμ. We are indebted to Eli Lilly and Co., Indianapolis, U.S.A., for data on one of our early preparations of *p*-aminobenzylpenicillin. They reported that the infrared spectrum of the material demonstrated that the lactam structure, amide grouping and other characteristic penicillin functions were present. Their ultraviolet-absorption spectrum analysis was in substantial agreement with that obtained in our Laboratories. They also showed that a basic group (pK_a 4·6) and an acid group (pK_a 2·6) were present.

Antibacterial properties. The antibacterial properties of *p*-aminobenzylpenicillin were compared with those of benzylpenicillin against several microorganisms. The results are given in Table 1.

The two penicillins seem to be about equally effective against staphylococci and streptococci, but it would seem that *p*-aminobenzylpenicillin is somewhat more effective (one to four times) against *Salmonella* organisms. This is of some interest in view of the activity of cephalosporin N against these organisms (Abraham, Newton & Hale, 1954; Heatley & Florey, 1953). According to the results of Heatley & Florey, and after correcting for impurities, cephalosporin N varies in its relative effectiveness as compared with benzylpenicillin from about 0·2 to 0·5 times for *Salmonella paratyphi* C to six times for *Salmonella enteriditis*. It appears, then, that *p*-aminobenzylpenicillin is about as effective as cephalosporin N against *Salmonella* when tested *in vitro*.

The amino group of *p*-aminobenzylpenicillin (pK_a 4·6) is not as basic as that of cephalosporin N (pK 9·8), but this lower basicity does not seem to alter appreciably its effectiveness against *Salmonella*. But, whereas cephalosporin N shows low activity against staphylococci and streptococci, *p*-aminobenzylpenicillin is about as active as benzylpenicillin against these organisms.

A clinical trial with synnematin B (cephalosporin N) showed that this drug was efficient for the treatment and control of typhoid fever and that it was more effective than chloramphenicol in

clearing *Salmonella typhi* from faeces (Benavides, Olson, Varela & Holt, 1955). However, the economic difficulties associated with its commercial production at present prevent its free use in medicine. It is possible that the more easily obtained *p*-aminobenzylpenicillin might be useful in the treatment of typhoid fever.

Derivatives of p-*aminobenzylpenicillin.* *p*-Aminobenzylpenicillin reacted with certain acid halides to form active derivatives which were less hydrophilic than was the parent compound and showed different solubility properties. It also reacted with *p*-nitrobenzenediazonium fluoborate to give a red, active reaction product. In each case reaction gave rise to an increase in the number of units of penicillin and also to a displacement of the position in which the activity appeared on chromatographic analysis.

EXPERIMENTAL

Microbiological assay. The penicillins were assayed by the Oxford cup-plate method with *Micrococcus pyogenes* var. *aureus*, strain 209 P, as the assay agent. The response curve obtained with benzylpenicillin was employed to assay *p*-nitrobenzylpenicillin, *p*-aminobenzylpenicillin and derivatives of *p*-aminobenzylpenicillin. Response curves for these penicillins differed slightly from each other as well as from that for benzylpenicillin.

Antibacterial assay. Solutions were assayed in Medium no. 3 (Grove & Randall, 1955), twofold dilutions being used. Each tube, containing 0·5 ml. of the penicillin dilution, was seeded with 0·5 ml. of a 10^{-4} dilution of a 24 hr. culture of the organism. Readings were made after incubation for 24 hr.

Chromatographic analysis. The method was that reported by Macmorine (1957) and found to give a good resolution of the polar penicillins.

Ultraviolet-absorption spectra. These were determined on a Beckman spectrophotometer model DK-2.

Hydrogenation. Stainless-steel cylindrical containers were used. These were equipped with a lid which could be bolted on and which was supplied with inlet and outlet openings and a pressure gauge. A Neoprene gasket was inserted between the container and the lid. Magnetic stirring bars covered with polytetrafluoroethylene (Teflon) were employed. Mild-steel containers are not satisfactory because, under the conditions which obtain, the β-lactam ring is opened by alcoholysis. The catalyst was 5% palladium on carbon (activity 980, Baker & Co. Inc., 113 Aston St., Newark 5, N.J., U.S.A.).

Fermentation. Vegetative inocula of *Penicillium chrysogenum* UV 15–4 for seeding medium in 20 l. fermenters were obtained according to Farrell (1953). The *p*-nitrophenylacetic acid was prepared according to Robertson (1932). The laboratory fermenter consisted of a standard 20 l. Pyrex bottle equipped for aeration, agitation, sampling, addition of anti-foam and temperature control. The equipment was described by Lane & Collingsworth (1948) and modified by Goldsmith (1951).

The components (%, w/v) of the medium used were: corn-steep liquor (50% of solids), 6·8; lactose 3·2; Cerelose (refined dextrose; Canada Starch Co., Cardinal, Ont.,

Table 1. *Comparative antibacterial titres of p-aminobenzylpenicillin and benzylpenicillin*

Organism	Lowest concn. (μg./ml.) preventing visible growth for 24 hr.		Activity of *p*-aminobenzylpenicillin
	p-Aminobenzylpenicillin	Benzylpenicillin	Activity of benzylpenicillin
Micrococcus pyogenes var. *aureus* (A.T.C.C. 653 P)	0·06	0·06	1
M. pyogenes var. *aureus* (resistant)	7·8	7·8	1
Streptococcus pyogenes (Dochez, N.Y. 5)	0·2	0·1	0·5
Salmonella typhi	7·8	15·6	2
	7·8	7·8	1
	3·9	15·6	4
S. enteriditis	1·95	7·8	4
	3·9	7·8	2
	3·9	7·8	2
S. paratyphi A	3·9	15·6	4
	3·9	15·6	4
	3·9	7·8	2
S. paratyphi B	3·9	3·9	1
	1·95	3·9	2
	3·9	3·9	1
S. paratyphi C	0·97	1·95	2
	0·97	3·9	4
	0·97	1·95	2
S. typhimurium	7·8	15·6	2
	7·8	15·6	2
	7·8	15·6	2
S. suispestifer	15·6	31·2	2
	7·8	31·2	4
	7·8	31·2	4

Canada), 0·45; $CaCO_3$, 0·9; KNO_3, 0·136; KH_2PO_4, 0·23; *p*-nitrophenylacetic acid, 0·182; lard oil, 0·25.

Production of p-*nitrobenzylpenicillin.* Fermentation medium (12 l.) was prepared in the fermenter and the pH was adjusted to 5·65 with 50% (w/v) NaOH. The medium was autoclaved (0·5 hr. at 121°), cooled and the bottle transferred to the fermentation bath. Seeding was carried out with 500–700 ml. of vegetative inoculum, and the temperature of the bath maintained at 26·5°, with an agitator speed of 400–500 rev./min. Samples were withdrawn at intervals for assay and pH determination. Antifoam (6% Alkaterge C; Commercial Solvents Corp., 260 Madison Avenue, New York 16, N.Y., U.S.A.) in lard oil was added when needed. An average of 26 ml. was necessary.

In 120 hr. titres of 2000–3400 units/ml. were reached. There was considerable evaporation of water during the course of the fermentation. The volume of filtered broth was 5–7 l./bottle.

To 13·82 l. of filtered broth (pH 7·5) was added 8 l. of commercial amyl acetate (mixed acetates; McArthur Chemical Co., 140 Liberty St., Toronto, Canada). The mixture was stirred vigorously and a solution of 20% (w/v) H_3PO_4 was added until the pH of the aqueous phase fell to 2·3. The 'amyl acetate' phase was separated in a Sharples centrifuge (laboratory model) and the aqueous phase washed with a small amount of 'amyl acetate'. The combined 'amyl acetate' phase (7·59 l.) was extracted with 0·4N-$KHCO_3$ soln. until the pH of the aqueous phase reached 6·8. The aqueous phase (281 ml.) assayed at 89 600 units/ml. It was treated with 1·5 g. of Darco KB charcoal (Atlas Powder Co. Can. Ltd., Brantford, Ont., Canada) and the carbon removed by filtration to give a concentrate of *p*-nitrobenzylpenicillin.

Ammonium sulphate was added (30 g./100 ml. of concentrate); the mixture was stirred for 15 min. and the precipitated ammonium salt of *p*-nitrophenylacetic acid was removed by filtration.

Crystalline potassium p-*nitrobenzylpenicillin.* The aqueous concentrate, after removal of precursor, was extracted at pH 2·2 with 0·5 vol. of 'amyl acetate' and the 'amyl acetate' phase was then extracted with 1·2N-$KHCO_3$ soln. until the pH rose to 7·1. The concentrated solution was again adjusted to pH 2 and extracted into an equal volume of 'amyl acetate'. The 'amyl acetate' phase was separated and dried with anhydrous Na_2SO_4. To the 'amyl acetate' concentrate was added, with vigorous stirring, a solution of KOH in butan-1-ol (40 g./l.) until the pH of the reaction mixture as measured with a Beckman meter (model G) reached 8·35. The mixture was put in the refrigerator (5°) overnight and the crystals were then removed by filtration and washed twice with butan-1-ol and three times with ether.

The yield from 13·82 l. of filtered broth was 5·45 g. of slightly yellow material assaying 1965 units/mg. Chromatographic analysis showed 96·7% of the activity in the G zone with 3·3% in the dihydro F zone and traces in the F and K zones. The product had an absorption maximum at 278 mμ in water (log ε = 4·01). $[\alpha]_D^{25} = +290$ in water (3·5 mg./ml.).

Crystalline potassium p-*aminobenzylpenicillin.* Potassium *p*-nitrobenzylpenicillin (10 g.) was added to a 1·6 l. stainless-steel reactor and to this was added a slurry of 5 g. of 5% palladium–charcoal catalyst in 36 ml. of water. Propan-2-ol (256 ml.) was added with stirring under N_2. The reactor was sealed, flushed with N_2 and filled with H_2 to a pressure of 28 lb./in.^{2}.

Stirring was started. The pressure dropped rapidly at first, reaching 15 lb./in.^{2} in about 20 min. and slowly thereafter until by 7 hr. very little H_2 was being taken up. The temperature of the reaction was kept at about 24° by means of a water-cooled jacket. After 7 hr. the reactor was flushed with N_2 and propan-2-ol (325 ml.) was added. The spent catalyst was removed by filtration and the filtrate was put in the refrigerator (5°) overnight. The next day there was a slight turbidity in the liquid. This was removed by filtration and the filtrate was distilled at about 20 mm. Hg and with a bath temperature of 40°.

The volume of liquid was reduced to about 60 ml., by which time well-formed needle-shaped crystals appeared. The crystals were removed by filtration, washed with propan-2-ol and dried at 100° *in vacuo*. The yield was 5·5 g. of slightly yellow-coloured material which assayed at 1000 units/mg. and gave 90% of the activity in the X zone on chromatographic analysis. When heated for 17 hr. at 100–110°, the product showed no loss of potency and no significant change in the zone at which the penicillin activity appeared on chromatographic analysis.

This product was recrystallized as follows: 1 g. was dissolved in water (1 ml.) and butan-1-ol (24 ml.) was added. The mixture was left in the refrigerator (5°) overnight. Next day the crystalline material was removed by filtration, washed with butan-1-ol, then with ether and dried. The yield was 0·62 g., which assayed at 1055 units/mg.

This product (0·6 g.) was dissolved in water (0·6 ml.) and propan-2-ol (7·5 ml.) was added. The crystalline precipitate (white needles) was removed by filtration, washed with propan-2-ol, then with ether and dried at 65° *in vacuo*. The yield was 0·38 g., which assayed at 1100 units/mg. and gave 100% of the activity in the X zone.

Reaction with benzoyl chloride. Potassium *p*-aminobenzylpenicillin (520 mg., 955 units/mg.) and $KHCO_3$ (200 mg.) were dissolved in water (2 ml.). Benzoyl chloride (0·15 ml.) was added and the mixture was shaken at room temperature until the odour of benzoyl chloride was no longer present. Water was added to a volume of 25 ml. This solution assayed at 28 600 units/ml. (91%, G zone). Thus from 496 600 units of *p*-aminobenzylpenicillin (almost completely in X zone) there was obtained 715 000 units (91%, G zone).

Reaction with 3:5-*dinitrobenzoyl chloride.* Potassium *p*-aminobenzylpenicillin (520 mg.) and $KHCO_3$ (250 mg.) were dissolved in water (2 ml.). To the solution was added, in small portions, with shaking and cooling, 3:5-dinitrobenzoyl chloride (300 mg.). Shaking was continued 0·5 hr. after evolution of gas had ceased, by which time a crystalline precipitate had formed. The mixture was placed in the refrigerator (5°) for 3·5 hr. and was then so thick it would not flow. Addition of water (18 ml.) gave a clear solution. Ethyl acetate (50 ml.) was added. The mixture was stirred vigorously and H_3PO_4 (10%, w/v) was added with stirring until the aqueous phase was pH 2·57. The ethyl acetate extract was shaken with 0·2N-$KHCO_3$ soln. (10 ml.) to yield an aqueous solution (10·5 ml., pH 7·8) which assayed at 67 550 units/ml. (100%, G zone). To this concentrate (4 ml.) water (4 ml.) was added and saturated aqueous potassium acetate soln. (1·9 ml.) to give an oily precipitate which crystallized on standing. This was separated by

centrifuging, washed with propan-2-ol and with ether and dried. Yield was 160 mg. of white powder, which assayed at 890 units/mg. (100 %, G zone) and which was contaminated with potassium acetate.

Reaction with p-nitrobenzenediazonium fluoborate. Potassium *p*-aminobenzylpenicillin (2 g.) and potassium acetate (3 g.) were dissolved in water (20 ml.). The solution was cooled to 0° and to it was added a cold solution of *p*-nitrobenzenediazonium fluoborate (1·22 g.) in water (100 ml.). The resulting viscous, red liquid was stirred for 2 hr. and then acetic acid (10 ml.) was added. In about 5 min. the viscous liquid changed to a jelly-like mass. This was left in the refrigerator (5°) overnight, was filtered, washed several times with water and pressed to a dark-red cake with a rubber dam. After drying there was obtained a brick-red powder (2·5 g.), which assayed at about 1400 units/mg. The activity of this material was found in an elongated zone between the F and K zones.

SUMMARY

1. With *p*-nitrophenylacetic acid as a precursor, *p*-nitrobenzylpenicillin was produced in fermentation media in titres as high as 3400 units/ml.

2. Crystalline potassium *p*-nitrobenzylpenicillin was isolated by the usual techniques employed in the purification of benzylpenicillin.

3. Hydrogenation of *p*-nitrobenzylpenicillin yielded *p*-aminobenzylpenicillin, which was isolated as the crystalline potassium salt. This is about as effective as cephalosporin N against *Salmonella* organisms *in vitro*.

4. *p*-Aminobenzylpenicillin reacts with certain acid chlorides and with *p*-nitrobenzenediazonium fluoborate to form active derivatives. These derivatives have greater activity than *p*-aminobenzylpenicillin when tested against *Micrococcus pyogenes* by the cup-plate method, and have altered solubility properties.

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